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(54) Cytotoxic conjugates for cancer therapy.

(57) Novel compositions and methods are provided for the treatment of cancer employing monoclonal antibodies (MoAbs) conjugated to a toxin. According to the present invention, MoAbs defining epitopes on either a tumor associated glycoprotein antigen of about 72 kD m.w. or on carcinoembryonic antigen conjugated to a ribosomal inhibiting protein, or the like, are employed either alone or in combination as cytotoxic agents in the treatment of various cancers including, but not limited to, colorectal carcinoma, ovarian carcinoma and osteogenic sarcoma.

Hydridomas XMMCO-791 and XMMCO-228 were deposited with the A.T.C.C. on August 14, 1986 and given A.T.C.C. Accession Nos. HB 9173 and HB 9174, respectively.



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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Y,D	INT. J. CANCER, vol. 33, 1984, pages 737-744; J. GALLEG0 et al.: "Preparation of four daunomycin-monoelonal antibody 791T/36 conjugates with anti-tumour activity" * Whole document *	1-29	A 61 K 39/395 A 61 K 47/00 C 12 N 5/00 C 07 K 15/00 C 12 P 21/00 // C 12 N 15/00 (C 12 P 21/00 C 12 R 1:91)
Y,D	BEHRING INST. MITT., no. 74, 1984, pages 108-112; M.J. EMBLETON et al.: "Experience in the preparation and experimental use of immunocytostatics" * Page 108, abstract *	1-29	
Y	US-A-4 590 071 (SCANNON) * Column 2, line 67 - column 5, line 60 *	1-29	
A	FEBS LETTERS, vol. 171, no. 1, June 1984, pages 31-35, Elsevier Science Publishers B.V.; M.R. PRICE et al.: "Characteristics of the cell surface antigen, p72, associated with a variety of human tumours and mitogen-stimulated T-lymphoblasts"		
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
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Place of search THE HAGUE		Date of completion of the search 02-08-1989	Examiner TURMO Y BLANCO C.E.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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CYTOTOXIC CONJUGATES
FOR CANCER THERAPY

CROSS-REFERENCE TO RELATED APPLICATION

5 This application is a continuation-in-part of
U.S. Application Serial No. 06/875,256, filed June 17,
1986, which is a continuation of U.S. Application
Serial No. 06/468,193, filed February 22, 1983, the
disclosures of which are hereby incorporated by
10 reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to
15 cancer therapy and, more particularly, to the treatment
of colorectal and ovarian carcinoma and osteogenic
sarcoma with cytotoxic conjugates of monoclonal
antibodies (MoAbs) and ribosomal inhibiting proteins
(RIPs).

20 Colorectal cancer is the second most common
cause of death from malignancy in the Western world.
The American Cancer Society estimates that there were
138,000 new cases of colorectal cancer and 59,900
patients died from the disease in 1985. The outlook
25 for patients with colorectal disease has remained
essentially unaltered over the last 30 years, with the
five-year survival being on the order of 30%. A major
factor contributing to this is the lack of effective
treatment for the disease once it has spread beyond the
30 bowel wall, since surgery during the early stages of
the disease offers the only prospect of cure.

Unfortunately, the majority of patients have
disseminated disease at the time of initial surgery,
primarily with overt or occult hepatic and lymph node.
35 metastases.

Despite numerous trials of chemotherapy, the only single agent shown to have any significant effect is 5-fluorouracil; its response rate is inadequate and treatment with it rarely influences the ultimate outcome. Combination chemotherapy and intrahepatic arterial infusion are being investigated but currently do not represent effective treatments.

Cancer of the ovary accounts for roughly 5% of all cancers in women and is the sixth leading cancer in women. Although surgery is curative if this lesion is detected early enough, the mortality associated with this disease has not improved appreciably in the last 25 years.

Ultrasound, laparoscopy or peritoneoscopy, and CAT scan are of limited value in the diagnosis of ovarian carcinoma. Serum markers such as carcinoembryonic antigen and placental alkaline phosphatase as well as some newly defined antigens are found in the blood of some patients with adenocarcinoma, although there is no universal marker. Surgery with biopsy is the only definitive way of diagnosing ovarian carcinoma.

Surgery is currently the only cure for ovarian carcinoma, and is only curative if the tumor has not spread. Radioisotope implants, x-ray irradiation, and chemotherapy are of limited use in the management of ovarian carcinoma.

The most important prognostic indication is the extent of spread of the tumor at the time of diagnosis and surgery. Stage I ovarian carcinoma (growth limited to the ovaries) has an overall 5-year survival rate of approximately 80%, Stage II (growth involving ovaries with pelvic extension) has a 5-year survival rate of 40%, Stage III (growth involving ovaries with extension to small bowel or omentum) has a 5-year survival rate of 10%, and Stage IV (distant

metastases) has a 5-year survival rate 37% of (1973-80), relatively the same as for 1960-63 (32%).

Imaging studies of ovarian carcinoma with radiolabelled MoAbs have been performed to a limited degree in human and animal systems. Tumors as small as 1 mm in diameter in mice bearing xenografts of human
5 ovarian cancer have been imaged. The same study also detected tumors in 8 out of 10 patients with ovarian cancer.

Osteogenic sarcoma (OS) is the most common primary bone tumor. Although surgery can be curative
10 if this lesion is detected early enough, the usual course of this disease in 80%-85% of patients is multiple pulmonary metastases and death within two years of diagnosis. These metastases are often present but usually not large enough to be seen at diagnosis of
15 the primary tumor.

Amputation is the treatment of choice for OS. Limb salvage procedures have been performed, such as en-block resection and prosthetic replacement. Overall survival for limb salvage is either as poor or worse
20 than with amputation.

Radiation treatment has not been shown to prevent OS metastases.

Most recent trials note survival rates of over 50% at 5 years for patients treated by widely
25 diverse adjunctive methods. The question is whether all of these widely diverse forms of treatment are effective or if there is a change in the natural history of the disease.

Imaging studies of OS with radiolabelled
30 MoAbs have been performed to a limited degree in human and animal systems. Human OS xenografts have been imaged in nude mice using an anti-OS MoAb labelled with ^{131}I .

Because of the extent of these three forms of
35 cancer, there is a need for new compositions and

methods to treat primary, recurrent and metastatic disease.

Description of the Relevant Literature

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SUMMARY OF THE INVENTION

Novel compositions and methods are provided to form the treatment of cancer employing monoclonal antibodies (MoAbs) conjugated to a toxin. According to the present invention, MoAbs defining epitopes on either a tumor associated glycoprotein antigen of about 5 72 kD m.w. or on carcinoembryonic antigen conjugated to a ribosomal inhibiting protein, or the like, are employed either alone or in combination as cytotoxic agents in the treatment of various cancers including, but not limited to, colorectal carcinoma, ovarian 10 carcinoma and osteogenic sarcoma.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphic illustration of the results obtained in the therapy of mice bearing 15 xenografts of osteogenic sarcoma 791T cells with XMMCO-791-RTA;

Figure 2 is a graphic illustration of the results obtained in the therapy of mice bearing xenografts of colorectal carcinoma C170 cells with 20 XMMCO-791-RTA at a dose of 5 mg/kg/day;

Figure 3 is a graphic illustration of the results obtained in the therapy of mice bearing xenografts of colorectal carcinoma C170 cells with XMMCO-791-RTA at a dose of 3 mg/kg/day; and

25 Figure 4 is a graphic illustration of the influence of free RTA and XMMCO-228-RTA on the growth of colorectal carcinoma LS174T cell xenografts.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention employs cytotoxic conjugates for the treatment of various forms of cancer, including colorectal carcinoma, ovarian carcinoma and osteogenic sarcoma. These novel conjugates are comprised of monoclonal antibodies or
5 binding fragments thereof, collectively termed immunoglobulins, bound to a cytotoxin. These compositions are administered to a cancer cell host in order to destroy cancer cells while doing minimal damage to normal tissue.

10 The immunoglobulins of the present invention are employed as targeting agents for directing cytotoxic agents to specific cancer cells within a cancer cell host. According to the present invention, immunoglobulins which define an epitope on a 72
15 kilodalton (kD) glycoprotein antigen and immunoglobulins which define an epitope on carcinoembryonic antigen (CEA) are employed as targeting agents. One or both of these antigens are present on a variety of cancers including, but not
20 limited to, colorectal carcinoma, ovarian carcinoma and osteogenic sarcoma.

These targeting immunoglobulins are bound to a variety of cytotoxic agents, generally ribosomal inhibiting proteins, more particularly, the A chain of
25 ricin or the A chain of abrin. The A chain of ricin has been successfully employed in cytotoxic conjugates as disclosed in U.S. Patent No. 4,590,071, the disclosures of which are hereby incorporated by reference.

The immunoglobulin and toxin are generally
30 bound by a covalent bond, more particularly a disulfide bond, but may be joined by any chemical bond which allows the toxin to travel to the target cell with the immunoglobulin. Other methods for achieving such a bond are well-known to those skilled in the art. The
35 only criteria is that the bond must be achieved in a

manner which does not significantly decrease the binding affinity of the immunoglobulin for its epitope.

The present cytotoxic conjugates may be administered to a cancer cell host either singly or in a cocktail containing two or more conjugate formulations. Cocktails are particularly important in the treatment of heterogeneous tumor cell populations wherein targeting of multiple antigens is critical.

The cytotoxic conjugates of the present invention may be administered to a cancer cell host by any convenient method. Pharmaceutical compositions employing the subject conjugates may be administered parenterally, i.e., intravenously, intraperitoneally, or the like. Thus, this invention provides compositions for parenteral administration which comprise a solution of pyrogen free, cytotoxic conjugates, or a cocktail thereof, dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine, or the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well-known filtration sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents, or the like, for example, sodium acetate, sodium chloride, potassium chloride, potassium chloride, calcium chloride, sodium lactate, etc.

The compositions of the present invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immune globulins and art-known lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that

lyophilization and reconstitution can lead to varying degrees of antibody activity loss and that use levels may have to be adjusted to compensate.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulation should provide a quantity of conjugate(s) of this invention sufficient to effectively treat the cancer cell host. The dose of conjugate or conjugate cocktail will vary widely, generally from about 0.01 mg/kg/day to 20.0 mg/kg/day, usually about 0.05 mg/kg/day to 10.0 mg/kg/day, and, more particularly, 0.05 mg/kg/day to 5.0 mg/kg/day.

Kits may also be supplied for use with the subject conjugates or cocktails. Thus, the subject compositions may be provided, usually in lyophilized form, either alone or in conjunction with additional chemotherapeutic agents for cancer therapy. These kits may include buffers, such as phosphate buffered saline, other inert ingredients, or the like. The kits may also include specific instructions including suggested protocols for the administration of the subject compositions to a cancer cell host.

The following examples are offered by way of illustration and not limitation.

EXPERIMENTAL

EXAMPLE I - XMMCO-791-RTA CONJUGATES

A. Production of XMMCO-791 Hybridomas

Balb/c mice (Bantin and Kingman, U.K.) were immunized with cultured 791T cells according to the following schedule:

1. Day 0: 1×10^7 cells were given intraperitoneally (IP) in Hank's balanced salt solution (HBSS);

2. Day 7: 1×10^7 cells were given IP in HBSS;

and

3. Day 21: 2×10^6 cells were given by intracardiac injection.

The 791T cell line used for immunization was obtained from Dr. Vera S. Byers, University of California, San Francisco. 791T is a human osteogenic sarcoma cell line isolated from the pulmonary metastasis of a 19-year-old Caucasian male. The cell line was characterized as malignant by its ability to grow as a tumor xenograft in nude mice, its lack of contact inhibition in culture and its abnormal nuclear and cytoplasmic morphology.

On day 26 after the initial immunization, spleen cells from immunized mice were aseptically removed. Following procedures as outlined elsewhere (Galfre *et al.*, Nature (1977) 226:550, which is incorporated by reference), 5×10^7 spleen cells were fused with an equal number of P3/NSI/1-Ag4-1 cells (A.T.C.C. Accession No. TIB-18) using polyethylene glycol 4000. The P3/NSI/1-Ag4-1 cell line is a non-secreting mouse myeloma cell line of Balb/c origin. This line is resistant to 8-azaguanine and lacks the enzyme hypoxanthine guanine phosphoribosyl transferase so that hypoxanthine- aminopterin-thymidine (HAT) medium becomes toxic, providing a convenient method of selecting unfused parental myeloma cells from the hybridoma population.

Cloning was performed by plating cells at low density in 0.3% agar in RPMI 1640 medium supplemented with 50 mg/ml gentomycin and hypoxanthine-aminopterin-thymidine (HAT) with 10% fetal calf serum, and overlaying a gel of 0.5% agar in the same medium. Spherical colonies were removed by Pasteur pipette. Recloning was performed by the limiting dilution technique, in which less than one hybridoma per three wells of a 96-well microtiter

plate was plated in the presence of 2×10^4 peritoneal exudate cells from unimmunized WAB/Not rats. Medium for regular maintenance was identical to the above, except that no aminopterin was included (HT medium).

The antibodies secreted by each clone were assayed for the appropriate specificity by radioimmune binding assay (Embleton *et al.*, Br. J. Cancer (1981) 43:582-87). One clone, designated XMMCO-791 was found to stably secrete monoclonal antibody; the antibody was determined to be of immunoglobulin class IgG2b.

Hybridoma XMMCO-791 is presently on deposit with the American Type Culture Collection (A.T.C.C.), 12301 Parklawn Dr., Rockville, M.D. 20852, U.S.A. The deposit was made on August 14, 1986, and given A.T.C.C. Accession No. HB 9173.

Specific pathogen free (SPF) Balb/c mice (Charles River) were used to culture the hybridoma intraperitoneally. Approximately 2×10^6 XMMCO-791 hybridoma cells in 0.5 ml of phosphate buffered saline (PBS) pH 7.2 were injected I.P. into pristane primed mice 7 to 21 days post priming. Priming was performed by injecting SPF Balb/c mice I.P. with 0.5 ml Pristane (2,6,10,14-tetramethylpentadecane). The resultant ascites fluid, collected 10-14 days post injection of hybridomas, contained 1.2 to 1.6 mg/ml of the antibody, as determined by double immunodiffusion.

The antibody in ascites fluid was purified following sterile filtration through a 0.22μ filtering system by loading onto a Staphylococcal protein A column (Staph protein A) and allowed to bind at 4°C , pH 7.5. The effluent was monitored spectrophotometrically until there was no further change of absorbance. The column was then subjected to pH gradient elutions (pH 5.0 and pH 3.0, 0.1 M sodium citrate buffer) and the monoclonal antibody collected. Purified antibody was stored frozen following aliquoting and quick freezing.

B. Antibody Binding to Cell Lines

The binding of XMMCO-791 to a large number of cultured human tumor cells and cultured human fibroblasts was compared by various assays to define tumor specific binding. These assays are detailed below. Studies show that there is little binding to normal fibroblasts, granulocytes, red blood cells, or lymphoblastoid cell lines; but there is significant binding to osteogenic sarcoma, colorectal and ovarian carcinoma. Studies described later demonstrate significant binding to freshly disaggregated human colorectal and ovarian carcinoma tumors.

1. ^{125}I Protein A-Binding Assay

Target cells in round-bottomed microtest plates were incubated on ice with hybridoma supernatant. The cells were washed and incubated for an additional 1 hour on ice with Protein A (Pharmacia) labeled with ^{125}I . The cells were washed and dried down. After spraying with a plastic film (Nobecutane), the wells were separated with a band saw and the bound ^{125}I measured in a gamma counter.

XMMCO-791 hybridoma supernatants were reacted with cultured cells derived from a range of human tumors and normal fibroblasts. Representative data are summarized in Table I. In tests with 36 malignant cell lines and 9 fibroblast cell lines, XMMCO-791 antibody reacted with 7/13 osteogenic sarcomas; 1/3 lung carcinomas, 1/1 prostate carcinomas, and 1/1 cervical carcinomas. 3/5 colorectal carcinomas also reacted strongly with binding ratios (BR) up to 33.40. Of the fibroblast lines, 1/9 reacted weakly (BR 2.14). Tests were carried out with fibroblasts derived from the donor of the tumor cell line 791T and these target cells did not react with XMMCO-791 (BR 0.99). Peripheral blood mononuclear cells were also negative.

TABLE I

Reactivity of XMMCO-791 Monoclonal Antibody
Against Cultured Human Cells
(Radioimmunoassay)

	Tumor Type	Cell Line	Mean cpm(\pm S.D.) with: P3NS1 supt.	XMMCO-791	Cpm ¹ increment	Binding ² Ratio
5	Osteogenic sarcoma	791T	480 \pm 100	10,965 \pm 378	10,485	22.84
	"	706T	275 \pm 44	685 \pm 260	410	2.49
	"	781T	553 \pm 9	1,014 \pm 105	461	1.83
	"	788T	364 \pm 142	17,044 \pm 1,646	16,680	46.82
	"	792T	358 \pm 55	443 \pm 18	85	1.23
10	"	803T	274 \pm 14	399 \pm 30	125	1.45
	"	805T	615 \pm 86	2,096 \pm 28	1,481	3.41
	"	836T	1,071 \pm 137	835 \pm 116	-236	0.78
	"	845T	250 \pm 30	1,373 \pm 73	1,145	5.58
	"	888T	860 \pm 104	926 \pm 85	66	1.08
	"	T278	794 \pm 36	16,490 \pm 404	15,696	20.77
	"	20S	1,542 \pm 213	4,689 \pm 341	3,147	3.04
	"	393T	676 \pm 20	1,110 \pm 123	434	1.64
15	Colon Carcinoma	HCT8	427 \pm 177	689 \pm 52	262	1.61
	"	HRT18	565 \pm 168	684 \pm 95	119	1.21
	"	HT29	522 \pm 101	3,474 \pm 177	2,952	6.66
	"	LS174T	266 \pm 48	3,786 \pm 203	3,520	14.23
20	"	HcLo	302 \pm 12	10,087 \pm 635	9,785	33.40
	Lung Carcinoma	A427	328 \pm 152	358 \pm 27	30	1.09
	"	A549	566 \pm 137	2,572 \pm 187	2,006	4.54
	"	9812	390 \pm 8	541 \pm 46	151	1.39
25	Breast Carcinoma	SK Br3	1,624 \pm 25	2,505 \pm 258	881	1.54
	"	578T	470 \pm 103	575 \pm 65	105	1.22
	"	734B	482 \pm 431	906 \pm 133	423	1.87
	"	MCF7	716 \pm 50	846 \pm 61	150	1.18
30	Melanoma	Nk1-4	285 \pm 36	258 \pm 70	73	1.25
	"	Mel-2a	418 \pm 105	568 \pm 13	150	1.36
	"	Mel-57	560 \pm 78	544 \pm 160	-16	0.97
	"	MeWo	232 \pm 36	228 \pm 36	-4	0.98
	"	RPMI5966	327 \pm 94	445 \pm 191	118	1.36

TABLE I (continued)

	Tumor Type	Cell Line	Mean cpm(\pm S.D.) with:		Cpm ¹ increment	Binding ² Ratio
			P3NS1 supt.	XMMCO-791		
5	Lymphoblastoid Line	Raji	668 \pm 354	844 \pm 347	176	1.26
		K562	1,086 \pm 171	905 \pm 115	-181	0.83
	Prostate Carcinoma	EB33	429 \pm 66	12,147 \pm 378	11,718	28.3
	Bladder Carcinoma	T24	285 \pm 40	429 \pm 28	144	1.50
10	Cervical Carcinoma	HeLa	258 \pm 18	14,257 \pm 658	13,999	55.52
	Ovarian Carcinoma	PA1	398 \pm 69	408 \pm 51	10	1.03
15	Fibroblasts	181 Sk	784 \pm 184	1,682 \pm 440	898	2.14
	"	788 Sk	1,033 \pm 374	927 \pm 125	-106	0.90
	"	791 Sk	403 \pm 39	399 \pm 54	-4	0.99
	"	803 Sk	350 \pm 39	362 \pm 28	12	1.03
	"	836 Sk	227 \pm 85	295 \pm 56	68	1.29
	"	860	1,165 \pm 38	1,624 \pm 347	459	1.39
	"	870	404 \pm 16	443 \pm 84	39	1.09
	"	618 Lu	500 \pm 51	538 \pm 82	38	1.09
20	"	74 BM	350 \pm 52	593 \pm 68	243	1.69
	Non-cultured cells:-	RBC	511 \pm 14	500 \pm 42	-71	0.86
	"	Mononuclear cells	410 \pm 112	492 \pm 115	82	1.20
25		SRBC	481 \pm 77	482 \pm 261	1	1.00

¹ CPM for XMMCO-791 minus cpm for P3NS1 supernatant.

² CPM for XMMCO-791 divided by cpm for P3NS1 supernatant. A binding ratio of >2 represents significant reactivity. These positive reactions are underlined.

2. Direct Binding Assay with ^{125}I -Labeled
XMMCO-791 Antibody

Tissue culture-derived target cells were aliquoted into round-bottomed microtest plates, sedimented by centrifugation, and incubated for 60 minutes with aliquots of ^{125}I -XMMCO-791. The cells
5 were washed and dried down. The wells were separated from the plate and bound radioactivity counted (Price MR, et al., 1982; Pimm MV, et al., 1982).

Direct binding of ^{125}I -labeled XMMCO-791 to a range of tumor cells is shown in Table II and this data
10 is summarized in Table III which expresses target cell binding of XMMCO-791 antibody relative to that with 791T cells. These tests demonstrate that ^{125}I -XMMCO-791 binds to osteogenic sarcomas 791T and 788T, and to HeLa cells. Binding of ^{125}I -XMMCO-791 to
15 other cell line derived tumor cells, including lung carcinoma (A427 and A549) and bladder carcinoma (T24), was approximately 10% of that with 791T cells.

3. Flow Cytometry

a. Direct Binding of FITC-Labeled XMMCO-791

20 Target cells were incubated with saturating doses of fluorescein isothiocyanate-labeled XMMCO-791 (FITC-XMMCO-791) and analyzed by flow cytometry. The binding of FITC-XMMCO-791 is expressed as fluorescence units/cell (Price MR, et al., 1983b; Gallego J. et al.,
25 1984; Roe R. et al., 1985).

TABLE II

Binding of ^{125}I -Labeled XMMCO-791 Monoclonal
Antibody to Cultured Human Tumor Cells

Exp.	Target Cells	^{125}I -antibody bound. (mean cpm \pm SD)
5	1	None
		Osteogenic sarcoma 791T
		Breast carcinoma SKBr3
		Colon carcinoma HCT8
		Lung carcinoma A427
		413 \pm 189
		6,301 \pm 605*
		772 \pm 61
		532 \pm 157
		741 \pm 152
10	2	None
		Osteogenic sarcoma 791T
		Osteogenic sarcoma 788T
		Osteogenic sarcoma 888T
		Bladder carcinoma T24
		Colon carcinoma HRT18
		Ovarian carcinoma PA1
		160 \pm 44
		3,265 \pm 425*
		3,356 \pm 163*
		452 \pm 25
		506 \pm 40
		571 \pm 60
		164 \pm 48
15	3	Osteogenic sarcoma 791T
		Osteogenic sarcoma 788T
		Osteogenic sarcoma 20s
		Osteogenic sarcoma 393T
		8,915 \pm 51*
		6,906 \pm 25*
		7,570 \pm 112*
		626 \pm 6

20 1.2×10^5 target cells were treated with 0.1 ml ^{125}I -labeled antibody preparation (50 ng protein) in replicates of four.

*Cpm is significantly higher than cpm with other cell lines within the same test as assessed by the Wilcoxon rank test.

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TABLE III

Direct Cell Binding of ^{125}I -labeled
XMMCO-791 Monoclonal Antibody

5	<u>Cell Line</u>	<u>Cell Type</u>	Percentage Binding ¹ of
			^{125}I -labeled Monoclonal Antibody
	791T	Osteogenic sarcoma	100
	788T	Osteogenic sarcoma	103
	888T	Osteogenic sarcoma	9
	HRT 18	Colorectal carcinoma	13
	HCT 8	Colorectal carcinoma	7
10	SK Br 3	Breast carcinoma	6
	A427	Lung carcinoma	11
	A594	Lung carcinoma	12
	HeLa	Cervix carcinoma	86
	PA1	Ovarian carcinoma	0
	T24	Bladder carcinoma	11
	RPMI 5966	Melanoma	13

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¹Binding of ^{125}I -labeled anti-791T/36 monoclonal antibody to various target cell lines is expressed as the percentage of the binding of labeled antibody to 791T tumor cells.

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Specificity is demonstrated by mixing unlabeled MoAb with FITC-labeled MoAb and demonstrating that the fluorescence per cell decreased. Titrating FITC-XMMCO-791 antibody against a fixed number of 791T cells indicated that maximum uptake of labeled antibody was obtained at approximately 0.1 μg antibody per 2×10^5 cells.

Table IV summarizes tests in which target cells derived from human tumor cell lines were reacted with FITC-XMMCO-791 and analyzed by flow cytometry. The antibody consistently reacted with osteogenic sarcoma 791T and 788T cells; these cells were used as standards for reference purposes. Ovarian carcinoma PA1 cells showed essentially no reactivity with XMMCO-791. All of the tumors listed bound significant amounts of FITC-XMMCO-791, although this was less than that observed with 791T cells. The C146, C168, and C170 cell lines were recently derived from surgical specimens obtained at the Department of Surgery, University Hospital, Queens Medical Centre, Nottingham, U.K. (Director, Professor J.D. Hardcastle). Since these tests were carried out with FITC-XMMCO-791 under saturating conditions, the number of antibody molecules bound/cell can be determined (Roe R., et al., 1985). Thus, colon tumor derived cells bound between 1.6×10^5 and 2.8×10^5 antibody molecules/cell compared with a value of 6×10^5 for osteogenic sarcoma 791T cells.

TABLE IV

Binding of FITC-XMMCO-791 Monoclonal Antibody
with Cultured Human Tumor Cells

5	<hr/>	
	Tumor Cell	Mean Fluorescence
	Osteogenic Sarcoma	Units/cell
	<hr/>	
10	791T	709.3, 722.4, 245
	788T	368.5
	Colon Carcinoma	
15	HCRT18	169
	LS174T	172.8, 187.6, 105.2
	C146	117
	C168	69
20	C170	88
	Ovarian Carcinoma	
25	PA1	<9
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b. Indirect Membrane Immunofluorescence Assay

Tumor cell lines were incubated with saturating amounts of XMMCO-791 monoclonal antibody, then washed and incubated with FITC-labeled rabbit anti-mouse Ig. The samples were analyzed by flow cytometry (Roe R., et al., 1985). Table V summarizes these tests with results expressed as fluorescence units/cell. The results showed the same pattern as with direct immunofluorescence: maximal binding with osteogenic sarcoma cell lines, intermediate with colon carcinoma cell lines, and low levels with ovarian, bladder, and breast carcinoma cell lines.

The binding of XMMCO-791 monoclonal antibody to tumor cells was also compared with that obtained with a range of other murine monoclonal antibodies. These include antibodies recognizing CEA epitopes (B14B8, 194.2 and 161) and other anti-colon carcinoma antibodies. These test, summarized in Table VI, show that XMMCO-791 binds to 791T cells, whereas none of the other antibodies were reactive with this cell line. Both XMMCO-791 and the anti-CEA antibodies reacted with colon tumor cells (LS174T, C170, HT29) and weakly with bladder carcinoma T24.

4. Complement Dependent Cytotoxicity

The complement dependent cytotoxicity of monoclonal antibody XMMCO-791 for target cells derived from cultured tumor cell lines was assayed using a 2 hour, ⁵¹chromium (⁵¹Cr) release assay (Price MR, et al., 1982).

Tumor cells (5×10^6) were labeled with ⁵¹Cr. The cells were aliquoted into microtest plates that contained either medium or diluted monoclonal antibody preparations. As a source of complement, normal rabbit serum was added to each well. Following incubation at room temperature for 2 hours, the supernatants were

collected and the percentag release of ^{51}Cr was
calculated for each test sample.

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TABLE V

Indirect Membrane Immunofluorescence
 Binding Activity of XMMCO-791
 Monoclonal Antibody as Assessed
 by Flow Cytometry

5	<u>Target Cell</u>	<u>Mean Fluorescence</u> <u>Units/Cell</u>
	Osteogenic Sarcoma	
	791T	1733, 369, 709, 667
	781T	1899
10	788T	338, 1572
	Colon Carcinoma	
	HCT8	335
	HT29	111
	LS174T	105, 173, 175
	HcLo	322
15	C146	351
	C168	184
	C170	302, 246
	Breast Carcinoma	
	BT474.3	80
20	Bladder Carcinoma	
	T24	82, 95
	Ovarian Carcinoma	
25	PA1	18, 10

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TABLE VI

Monoclonal Antibody Binding to Tumor Cells:
Indirect Flow Cytometry Assay

<u>Target Cell</u>	<u>Reagent</u>	<u>Fluorescence units/cell</u>
Osteogenic Sarcoma 791T	----	30.0
	NMIg	28.4
	XMMCO-791	3380.0
	SV2372	20.0
	19G415	45.9
	B14B8	31.6
	194.2	35.2
	FX13D4	65.7
Colon Carcinoma LS174T	----	43.1
	NMIg	53.4
	XMMCO-791	209.1
	B14B8	1325.5
	FX13D4	957.0
	194.2	1212.9
C170	----	25.1
	NMIg	30.1
	XMMCO-791	302.2
	SV2372	27.9
	19G4.15	43.8
	B14B8	59.2
	161	36.2
	45.2D9 C4	30.3
HT29	----	41.4
	NMIg	47.0
	XMMCO-791	111.2
	161	252.8
	194.2	47.6
	FX13D4	56.0
HCT8	----	36.9
	NMIg	31.1
	XMMCO-791	335.2
	161	128.4
	194.2	37.3
	FX13D4	926.4

TABLE VI (Continued)

	<u>Target Cell</u>	<u>Reagent</u>	<u>Fluorescence units/cell</u>
5	Bladder Carcinoma T24	----	41.4
		NMIg	47.0
		XMMCO-791	82.0
		161	81.6
		194.2	44.6
		FX13D4	60.0
10	Breast Carcinoma BT474.3	----	70.4
		NMIg	76.5
		XMMCO-791	196.5
		B14B8	271.2
		FXT3D4	87.9
		194.2	253.9
15		161	281.4

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Table VII summarizes tests on the complement mediated cytotoxicity of XMMCO-791 monoclonal antibody preparations. XMMCO-791 antibody was cytotoxic for osteogenic sarcoma derived cell lines 791T, 788T, 20S, and 278T, prostate carcinoma derived cell line EB33, and for HeLa cells, but not for ovarian carcinoma derived cell line PA1 or lung carcinoma A549 cells.

C. Antibody Binding to Human Malignant Cells and Tissues

1. Radioimmunoassay of XMMCO-791 Binding to Tumor Membranes

Monoclonal antibody XMMCO-791 binding to membrane preparations derived from surgically removed tumors was determined using a solid phase radioimmunoassay.

Tumor membrane preparations (ENM) were made by homogenizing fresh surgically removed tumor. Nuclei and cell debris were removed by centrifugation (600 g) and the ENM material sedimented at 105,000 g. ENM preparations attached to polyvinylchloride microtiter plates were used to assay antibody binding, this being detected by uptake of ^{125}I -labeled rabbit anti-mouse IgG F(ab')₂ (Brown A., et al., 1983) (Table VIII). Positive tumor:nontumor ratios were obtained in 3/5 tumors.

2. Flow Cytometry

Binding of monoclonal antibody XMMCO-791 to malignant cells derived from primary and metastatic human tumors has been determined by flow cytometry. For this purpose, tumor cells were brought into suspension by collagenase treatment, and reacted with either XMMCO-791 or with control murine monoclonal antibodies. Cell bound immunoglobulin was then detected with FITC-conjugated rabbit anti-mouse Ig

TABLE VII

Complement Dependent Cytotoxicity of XMMCO-791
Monoclonal Antibody

5	Cell Line	Cell Type	Percentage Cytotoxicity (Mean \pm SD) Antibody at Protein Concentrations (μ g/ml)		
			125	25	5791T
	791T	Osteogenic sarcoma	62 \pm 2 (1 \pm 1)*	41 \pm 2	12 \pm 2
10	788T	Osteogenic sarcoma	43 \pm 1 (1 \pm 1)	25 \pm 1	20 \pm 2
	278T	Osteogenic sarcoma	50 \pm 3 (1 \pm 1)	12 \pm 1	4 \pm 1
	20S	Osteogenic sarcoma	32 \pm 2 (1 \pm 1)	1 \pm 1	1 \pm 1
15	EB33	Prostate carcinoma	41 \pm 6 (1 \pm 1)	4 \pm 1	3 \pm 1
	A549	Lung carcinoma	0 \pm 1 (0 \pm 0)	0 \pm 0	0 \pm 0
20	HeLa	Cervix carcinoma	45 \pm 4 (1 \pm 1)	28 \pm 2	21 \pm 2
	PA1	Ovarian carcinoma	0 \pm 1 (1 \pm 1)	0 \pm 0	0 \pm 1

25 * Percentage cytotoxicity with heat inactivated complement.

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TABLE VII

Radioimmunoassay of Binding of
XMMCO-791 to Colon Carcinoma and
Normal Colon Membran Preparations

5	Patient	XMMCO-791 antibody binding (cpm \pm SD) to membrane preparations from:		
		Colon Tumor (T)	Normal Colon (N)	T:N ratio
	109	842 \pm 52	85 \pm 41	9.9:1
	147	620 \pm 48	307 \pm 31	2.0:1
10	162	318 \pm 11	242 \pm 34	1.3:1
	167	2074 \pm 71	286 \pm 19	7.2:1
	170	386 \pm 54	360 \pm 51	1.0:1
15	Membrane bound 791T/36 detected by 125 I-labeled rabbit F(ab') ₂ anti-mouse IgG.			

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and analyzed by flow cytometry. The analysis was restrict d to the size range of malignant cells by appropriate forward angle scatter gating (Durrant LG, et al., 1986a).

XMMCO-791 antibody reaction with tumor cells derived from a primary colon carcinoma and from liver and lymph node metastases was tested. Significant binding of XMMCO-791 with tumor cells from both the primary and metastatic tumor was shown. For comparison, binding tests were carried out at the same time with two other murine monoclonal antibodies, C194 and C14|1|46; these MoAbs recognize CEA and Y hapten (Brown A., et al., 1983), respectively. Overall, the reaction with XMMCO-791 was comparable with that obtained with these antibodies.

The results of flow cytometry studies on binding of XMMCO-791 MoAbs to freshly disaggregated ovarian tumors are shown in Table VIIa. The XMMCO-791 MoAb bound to the cell surface of all but one ovarian tumor. Intensity of staining was weak (MLF<100) in one tumor, moderate (MLFs 100-1000) in 8 and strong (MLF>1000) in one.

TABLE VIIIa

Flow Cytometric Analysis of Binding
of 791T/36 to the Cell Surface of
Freshly Disaggregated Ovarian Tumors

Indirect Immunofluorescence on Ovarian Tumor Cells
(Mean Linear Fluorescence, MLF):

	Ov1	Ov2	Ov3	Ov4	Ov5	Ov6	Ov9	Ov9	Ov10 (met) ¹	Ov11	Ov12
Antibody											
791T/36	138	61	105	138	135	105	249	456	94	155	1808
Normal mouse IgG	36	54	20	21	41	20	51	58	17	51	133

(1) Peritoneal metastases of primary tumor Ov9

3. Characterization of XMMCO-791 Binding Antigen
in Human Primary Colon Carcinoma Tumors

As will be described later, studies carried out at University of Nottingham, U.K., with appropriate regulatory approval, demonstrated that the MoAb XMMCO-791, when injected in vivo, localizes in primary and metastatic human colon cancers. Thus, studies were carried out to identify the antigen to which the antibody is bound in vivo.

Briefly, 200 μ g ^{131}I -XMMCO-791 containing 2 mCi ^{131}I was injected into patients with primary colon carcinomas, and 1-6 days later normal colon tissue and colon tumors were resected. Tissues were homogenized and the fibrous material was extracted. The extract was then mixed with Affi Gel 10 (BioRad Laboratories, Richmond, CA), to which affinity purified goat anti-mouse IgG antibody had been linked. The pellets contained the bound ^{131}I -labeled material from the tissue extract. This was reacted with ^{125}I to label tissue protein.

After washing, the Affi Gel pellet was treated with sodium isothiocyanate to release bound ^{131}I -labeled XMMCO-791 antibody, either free or complexed with antigen.

After purification by gel chromatography, the extract was incubated with Sepharose-Protein A to isolate Ig containing material. These products were removed from the gel and SDS-Polyacrylamide-Gel-Electrophoresis (SDS-PAGE) was carried out. The results are summarized in Table IX.

The apparent molecular weights of two of the bands at 60 kD and 25 kD corresponded to those for heavy and light chains of the XMMCO-791 IgG2b antibody. The third band at 72 kD corresponded to that of the XMMCO-791-defined antigen isolated from 791T cells. The conclusion from the studies is that following infusion of XMMCO-791 antibody into colorectal

carcinoma patients, the antibody localizes within the tumor by binding to tumor-associated antigen of molecular weight (72 kD) identical to that found on 791T cells, the original immunogen.

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TABLE IX

Localization of ^{131}I -Labeled XMMCO-791
Monoclonal Antibody in Primary
Human Colorectal Carcinoma

5	Clinical Data	Patient 1	Patient 2	Patient 3
10	Tumor Type:	Rectal adenocar- cinoma	Caecum adenocar- cinoma	Sigmoid adenocar- cinoma
10	Duke's Stage:	B	B	C
10	Degree of Differentiation:	moderate	well	moderate
10	Tumor size (mm)	30 x 20	35 x 35	85 x 60
15	Patient Infused with:	^{131}I -XMMCO-791 + ^{125}I -IgG _{2b}	^{131}I -XMMCO-791	^{131}I -XMMCO-791
20	Period Between Antibody Infusion and Tumor resection (days):	1	3	3
20	Distribution of radioactivity of dose of ^{131}I -XMMCO- 791 per g of tumor tissue:	0.008	0.003	0.004
25	Tumor:Normal Tissue Ratio:	3.3:1(^{131}I) 1.2:1(^{125}I)	2.0:1(^{131}I)	1.8:1(^{131}I)
30	Radioactivity (^{131}I) Recovered in Fibrous Material from tumor tissue:	87	83	74

D. Antibody Binding to Normal Human Cells

1. R activity of XMMCO-791 Monoclonal Antibody
with Human Peripheral Blood Mononuclear Cells
(PBMC) Analyzed by Flow Cytometry

a. Lymphocytes

Using direct and indirect immunofluorescence
5 assays, XMMCO-791 antibody was shown to react strongly
with phytohemagglutinin (PHA) (Sigma) stimulated
lymphocytes from peripheral blood and negligibly with
resting lymphocytes. Maximum binding was observed
after 3 days culture with PHA (5 µg/ml), coinciding
10 with maximum DNA synthesis. Comparable results were
obtained using concanavalin A (Con A) (10 µg/ml) as
mitogen (Table X).

XMMCO-791 antibody precipitated a protein of
apparent molecular weight of 72,000 from cells cultured
15 3 days with PHA. No other bands at 72,000 were
precipitated from other cultures of PBMC and no 72,000
molecular weight material was precipitated from
PHA-stimulated PBMC using normal mouse immunoglobulin,
rather than XMMCO-791 antibody.

20 b. Blood Granulocytes

Binding of XMMCO-791 to peripheral blood
granulocytes has been determined using an indirect flow
cytometry assay.

Table XI summarizes a series of tests of 9
25 lots of XMMCO-791 before and after ultracentrifugation
with blood granulocytes from 2 donors. The binding of
XMMCO-791 to granulocytes was compared with that of
supernatant made by the parental myeloma line,
P3/NSI/1-Ag4-1. 8/9 lots showed negative binding. One
30 lot showed moderate binding which became negative after
centrifugation.

TABLE X

Binding of FITC-labeled XMMCO-791 Antibody to
Mitogen-Stimulated PBMN Cells as Assessed by
Quantitative Flow Cyt fluorimetry

5	Cell Subpopulation Analyzed	Percentage of cells in subpopulation analyzed (mean \pm SD)	Number of FITC-XMMCO- 791 antibody molecules binding/cell ($\times 10^{-3}$) (mean \pm SD)
10	Small lymphocytes from non-stimulated PBMN cell preparations	93.4 \pm 3.8 (n = 18)	<0.1 (n = 18)
15	Lymphoblasts from PBMN cell preparations cultured with PHA for 3 days	45.5 \pm 22.2 (n = 17)	1.1 \pm 0.4 (n = 17)
20	Lymphoblasts from PBMN cell preparations cultured with Con A for 3 days	60.6 \pm 12.0 (n = 6)	0.9 \pm 0.5 (n = 6)

The number of determinations (n)
is given in parenthesis

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TABLE XI

Monoclonal Antibody XMMCO-791
Binding to Blood Granulocytes.
Indirect FACS Analysis Before
and After Centrifugation

5	<u>No.</u>	<u>Sample</u>	<u>Mean Fluorescence Intensity/Cell</u>			
			<u>Donor 1</u>		<u>Donor 2</u>	
			<u>Before</u>	<u>After*</u>	<u>Before</u>	<u>After</u>
	1	3929	83.9	27.7	67.0	28.6
	2	4110	24.2	24.7	21.4	25.4
10	3	3619	34.8	35.8	34.9	32.1
	4	4086	25.0	22.5	31.1	29.1
	5	4056	24.1	25.1	25.4	31.1
	6	3984	27.6	25.1	45.2	32.3
15	7	50108	25.1	22.7	28.9	28.6
	8	4110	---	---	28.6	28.5
	9	P3NS1	19.8		19.2	

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* Ultracentrifuged 105,000g/60 minutes

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E. Binding to Other Normal Human Tissues Measured By Immunoperoxidase

XMMCO-791 was extensively evaluated for crossreactivity with normal tissues using the indirect immunoperoxidase conjugate technique. As a control, tissues were simultaneously tested with a purified murine MoAb in the same concentration. The control immunoglobulins used for these purposes were an IgG2 mouse myeloma protein (UPC 10, Litton Bionetics, Kensington, MD) or a MoAb with reactivity to sheep red blood cells (SRBC).

Fresh tissue slices (2-3mm thickness and up to 2.0 cm in width) was generously coated with Optimal Cutting Temperature Compound (OCT), (Ames Co., Elkhart, Indiana) and wrapped in aluminum foil. The specimen was "snap" frozen in liquid nitrogen-cooled isopentane (2-methylbutane, VWR, Norwald, CA) at controlled temperature (-120 to -150°C) for 10-15 seconds. The tissue was kept frozen for long-term storage at -70°C, or for short-term storage at -20°C, in a tightly sealed container to prevent evaporation and drying of tissues.

Frozen tissues were allowed to equilibrate slowly overnight by placing in a -35°C cryostat prior to cutting the frozen sections. Cut sections were dried overnight at room temperature prior to staining.

A circle was etched on each slide around the tissue using a diamond pencil. Slides were then fixed in acetone for 1 minute, followed by a 10 minute PBS wash. Excess fluid was removed and the primary antibody applied for one hour while making sure that the tissue was covered and not allowed to dry out, followed by a 10 minute PBS wash.

Excess fluid was removed and peroxidase-conjugated goat anti-mouse IgG (Tago/product code 6450) (diluted 1:10 in PBS) applied for 30 minutes, followed by a 10 minute PBS wash.

The slides were developed in aminoethylcarbazole (AEC), (0.5 ml AEC stock 120 mg AEC in 15 ml dimethylformamide), 9.5 ml acetate buffer (79 ml of 0.1 M sodium acetate, 21 ml of 0.1 N acetic acid), and 0.05 ml 3% H₂O₂) for 5 minutes, followed by a 5 minute H₂O wash. The slides were then counterstained with fresh Mayer's Hemtoxylin for 5 minutes, followed by a 5 minute wash in running tap water and coverslipped with glycerine mounting media. The following is a summary of the results:

- 10 - Throughout the study, connective tissue (ground substance) was positive.
- The vascular endothelium of capillaries and venules in varying tissues were mildly positive but not all venules were positive in any tissue.
- 15 - Lung tissue from 2/4 autopsy specimens showed some reactivity with about 10% of the alveolar epithelium/macrophages in each of the two specimens; most reactivity was in macrophages.
- Kidney tissue showed faint glomeruli staining in 10% of the sample in 1/4 autopsy specimens.
- 20 - Skin showed staining in the lumen of ducts in 2/4 autopsy specimens.
- Colon showed slight staining of glandular epithelium in both colon specimens tested.
- 25 - Fetal tissue showed some staining in alveolar and bronchial epithelium of the lung; some positive tubule lumen in the kidney.
- There was no staining (except for connective tissue and venules) in:

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Thyroid	Liver	Heart
Breast	Adrenal	Muscle
Spinal cord	Nerve	Thymus
Testes	Diaphragm	Uterus
Esophagus	Pancreas	Aorta
Spleen	Brain	

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Note: Granulocytes will appear positive in both control and test tissue because of endogenous peroxidase.

10 F. Identification of the Cell Antigen Binding
XMMCO-791

1. Osteogenic Sarcoma Derived 791T Cells

791T cells were surface-labeled with ^{125}I by lactoperoxidase catalyzed radioiodination and lysed. A
15 lysate containing ^{125}I -labeled membrane protein was obtained. Sodium dodecyl sulfate (SDS) and XMMCO-791 MoAb were added to aliquots of this cell lysate, followed by Sepharose-Protein A beads. The beads were washed and final pellets were suspended in reducing gel
20 sample buffer. After incubation, supernatants were analyzed by polyacrylamide gel electrophoresis. Gels were stained for protein with Coomassie blue and, after drying, autoradiographed.

The antigen precipitated by the MoAb
25 XMMCO-791 from detergent lysates of ^{125}I -labeled 791T cells migrated in SDS-PAGE slab gels with an apparent molecular weight of 72,000. Both reduced and non-reduced samples gave equivalent results, indicating that the antigen is a monomeric cell surface protein.
30 The relatively broad band suggests that the antigen may be glycosylated, with microheterogeneity in the saccharide residues producing the wide band. When immunoprecipitates were treated with neuraminidase, the apparent molecular weight was reduced to 55,000, indicative of extensive glycosylation. Since
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microheterogeneity in the band of precipitated material was lost following neuraminidase treatment, this indicated that microheterogeneity was attributable to variations in sialic acid content.

That the gp72 antigen is glycosylated was confirmed by labeling growing tumor cells with ^3H

5 glucosamine and again precipitating a 72,000 molecular weight antigen which was radiolabeled.

2. Other Human Tumor Cell Lines

Seven cell lines known to express the antigen recognized by XMMCO-791 were selected. Four of these
10 were osteogenic sarcomas, two were colon carcinomas, and one was a prostate carcinoma cell line. Negative controls included lung carcinoma, breast carcinoma, and malignant melanoma cell lines.

These 10 human tumor cell lines were surface-labeled with ^{125}I , lysed with detergent and subjected to radioimmunoprecipitation using either the XMMCO-791 monoclonal antibody or normal mouse immunoglobulin. The immune precipitates were isolated using Sepharose-Protein A and analyzed by SDS-PAGE and
20 autoradiography. The XMMCO-791 antibody precipitated a protein from 791T cells of apparent molecular weight of 72,000 in accordance with previous findings. Five other cell lines contained protein material of apparent molecular weight of about 72,000 that was also precipitated by the XMMCO-791 antibody. Colon carcinoma cell
25 line HT29 gave anomalous results, since despite repeated testing, no protein was precipitable with the XMMCO-791 antibody. The cell lines A549, B55, and RPMI 1566, which are unreactive with the XMMCO-791 antibody,
30 failed to express a 72,000 dalton antigen in these immunoprecipitation studies.

It is evident from these studies that the precipitates from the other cell lines which bound XMMCO-791 MoAb were not identical to that from the 791T
35 cell line, although proteins of 72,000 daltons were

detected in each case. These differences may be due to antigen heterogeneity (characteristic of carbohydrate-rich glycoprotein antigens) and/or variations in the specific activity of the antigens with respect to radiolabeling. The cell line EB33 clearly showed two distinguishable components of 72,000 and 55,000 daltons that were precipitated by the XMMCO-791 antibody.

a. Effect of Neuraminidase Treatment

Neuraminidase reduced the apparent molecular weight of the precipitate antigens in five of the six cell lines tested to a uniform value of 55,000 daltons.

These results indicate, first, that the precipitated labeled protein antigens are glycosylated and, second, that their molecular heterogeneity is attributable to differences in sialic acid content. The protein precipitated from the colon carcinoma cell line HcLo was apparently not affected by treatment with neuraminidase.

b. Effect of Chymotrypsin Treatment

Radiolabeled immune precipitates were treated with chymotrypsin and analyzed by SDS-PAGE and autoradiography to compare the XMMCO-791 defined antigens with respect to their susceptibility to proteolysis. The XMMCO-791-defined antigen proved to be very resistant to degradation, the major chymotryptic product in each case being a polypeptide with an apparent molecular weight of 47,000. This would indicate that each of the antigens immunoprecipitated with the XMMCO-791 antibody have at least one chymotryptic site in common. The major 47,000 molecular weight polypeptides from the six tumor cell lines examined did not display the molecular heterogeneity associated with the non-enzyme-treated XMMCO-791-defined antigens. This would imply that the portion(s) of the molecule exhibiting a variable content of sialic acids was (were) removed by proteolysis with chymotrypsin. It may be noted that the antigen

from the cell line HcLo was not anomalous with respect to its susceptibility to chymotrypsin. This contrasts with its apparent resistance to neuraminidase since the major polypeptide generated was of 47,000 dalton molecular weight.

3. Effect of Papain on the gp72 antigen

5 Previous studies have demonstrated that 791T cells treated with papain no longer bind XMMCO-791 antibody, indicative of the degradation or release of XMMCO-791-defined antigen. To define the action of papain more precisely, 791T cells were radiolabeled
10 with ^{125}I and treated with papain. After centrifugation, the solubilized extract of cell membrane proteins and glycoproteins was subjected to radioimmunoprecipitation using XMMCO-791 antibody and Sepharose-Protein A, and the immune precipitates were
15 analyzed by SDS-PAGE and autoradiography. The results indicate that papain digestion released an antigenically active protein fragment of apparent molecular weight of 53,000. Neuraminidase treatment of the immune precipitate prepared as above effected a
20 further reduction in apparent molecular weight to 50,000, indicating that this papain fragment was glycosylated and carried sialic acid residues.

4. Purification of the ^{125}I Labeled gp72 Antigen

To develop a purification procedure for the
25 gp72 antigen, 791T cells were surface labeled with ^{125}I and lysed with detergent. After centrifugation and dialysis, the soluble extract was fractionated by immunoadsorbent chromatography by passage through two non-specific adsorbent columns (Sepharose 4B and Affi
30 Gel 10-linked mouse IgG) and a specific immunoadsorbent column (Affi Gel 10-linked XMMCO-791 monoclonal antibody) connected in series. The XMMCO-791 antibody immunoadsorbent was eluted and rechromatographed.

The purified antigen consisted of a major
35 band at 72,000 daltons. There was also a minor band of

slightly lower molecular weight; it may be due to carbohydrate heterogeneity.

5. Binding of ^{125}I -Labeled gp72 to Lectins

The binding of ^{125}I -labeled XMMCO-791-defined antigen to lectins was investigated. After incubation of aliquots of the antigen preparation (1×10^4 cpm) with Sepharose-wheat germ agglutinin (WGA), Sepharose-Con A and Sepharose-lentil lectin, bound glycoproteins were eluted and analyzed by SDS-PAGE and autoradiography. The material eluted from Sepharose-WGA migrated with an apparent molecular weight of 72,000, indicating the presence of N-acetyl-glucosamine in this glycoprotein. Equivalent material was also bound to Sepharose-Con A, but no bands were seen with eluates from Sepharose-lentil lectin or Sepharose alone. Of the radioactivity recovered in the eluates from these columns, 69% was present in the fraction bound and eluted from Sepharose-WGA, 49% was bound and eluted from Sepharose-Con A, 23% from Sepharose-lentil lectin and 9% from Sepharose alone.

20 6. Conclusions

XMMCO-791 MoAb reacts with the osteogenic sarcoma cell line 791T used for initial immunization. The XMMCO-791-defined epitope on this cell line is expressed on a protein of apparent molecular weight 72,000. The MoAb also reacts with cell lines other than this one. An investigation was performed to determine whether the epitope occurred on similar molecules on these other cell lines. It was concluded that molecules expressing this epitope do exist on other tumor cell lines. These molecules are glycoproteins that have different molecular weights. This difference is attributable to varying degrees of sialylation. There are no apparent differences in the polypeptide "backbone" of these antigenic molecules. Radiolabeled immunoprecipitates, prepared with the XMMCO-791

antibody, from three osteogenic sarcoma cell lines (2 OS, 788T, and 278T), the prostate carcinoma EB33 and the colon carcinoma HcLo each contained a protein with a molecular weight of 72,000 as the major constituent and, in some cases, material of lower weight. By neuraminidase treatment of the immune precipitates,

5 this heterogeneity was shown to be due to variations in sialic acid content of the antigens since; in five of the six cell lines tested, such treatment produced homogeneous material of apparent molecular weight 55,000. Chymotrypsin treatment of the immune precipitates

10 produced in each instance a major polypeptide of molecular weight 47,000 which displayed no microheterogeneity. Immunoabsorbent-purified antigen from 791T cells was shown to bind strongly to Sepharose-concanavalin A, confirming the glycoprotein nature of this antigen.

15 These results are summarized in Table XII.

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TABLE XII

REACTIVITY OF THE MONOCLONAL ANTIBODY XMMCO-791
WITH CULTURED HUMAN CELLS

5	Target Cell	Antibody reactivity with viable target cells:		Apparent molecular weight ($\times 10^{-3}$) of target antigen (SDS-PAGE) ³	
		Cell binding activity ¹	Antibody molecules bound per cell ($\times 10^{-4}$) ²	Before neuramin- idase	After neuramin- idase
10	Osteogenic Sarcomas:				
	791T	+++	220	72	55
	788T	+++	160	72	55
	278T	++	51	72	55
	20S	+	53	72	55
15	Carcinomas:				
	HcLo (colon)	+++	ND	72	72
	HT29 (colon)	++	ND	No precipitate	NR
	EB33 (prostate)	+++	35	72;55	55
	A549 (lung)	---	ND	No precipitate	NR
20	B55 (breast)	---	ND	No precipitate	NR
	Melanoma:				
	RPM1				
	1566	---	<2	No precipitate	NR

¹Cell-binding of XMMCO-791 antibody was determined using the indirect ¹²⁵I-Protein A binding assay (Embleton MJ, et al, 1981). +++ represents binding ratios >10. ++ represents binding ratios of 5-10. + represents binding ratios of 2-5 and --- represents binding ratios <2 where binding ratio = (mean cpm bound to cells treated with XMMCO-791 antibody)/(mean cpm bound to cells treated with P3NS1 spent medium).

²Quantitative binding of ¹²⁵I-labeled XMMCO-791 antibody determined by Price MR, et al. (1982a).

³(Price MR, et al, 1983b)

ND, not determined

NR, not relevant

G. Biological Properties of XMMCO-791

1. Complement Dependent Cytotoxicity

Complement dependent cytotoxicity of monoclonal antibody XMMCO-791 was assayed in a 2 hour ⁵¹Chromium release assay. It was found that only rabbit serum served as an effective complement source for lysis of target cells (Table XIII).

2. Antibody Dependent Cellular Cytotoxicity (ADCC)

Experiments were carried out to determine whether XMMCO-791 mediated ADCC reactions (Flannery et al., Eur. J. Cancer Clin. Oncol. (1984) 20:791-98). ⁵¹Cr-labeled target cells (791T, and the human erythroleukemic cell line K562) were incubated with appropriate dilutions of antibody and aliquoted into microtiter wells. Appropriate numbers of human mononuclear cells were added.

XMMCO-791 is not able to mediate ADCC against either 791T cells or K562 cells at concentrations of antibody ranging from 10 to 0.1 µg/ml and with effector:target cell ratios ranging from 0.8:1 to 3:1.

3. In Vivo Inhibition of Tumor Growth

The effect of XMMCO-791 monoclonal antibody alone on the in vivo growth of human tumors was assessed using xenograft systems. 791T cells were implanted subcutaneously into mice immunodeprived by thymectomy and whole body irradiation. Mice were treated with XMMCO-791 antibody starting 1 to 5 days after 791T implantation and were treated at intervals thereafter.

Three injections of either 40 µg or 80 µg did not modify growth of 791T xenografts. In further tests, doses of up to 181 µg/kg body weight (given in 9 divided doses) have not suppressed growth of 791T xenografts.

TABLE XIII

Complement-Dependent Cytotoxicity of Anti-791T Monoclonal Antibody XMMCO-791
Screen of Complement Sources

Serum Source of Complement*	% Cytotoxicity Against				
	791T/Cells Treated With		PHA-Blasts Treated With		
	Medium	XMMCO-791	Anti-791T/48+	W6/32++	Medium
Rabbit	-0.3 ± 0.3 (-0.2 ± 0.1)	53.8 ± 4.7 (0.7 ± 0.5)	29.1 ± 2.8 (0.4 ± 0.6)	46.1 ± 5.3 (0.9 ± 0.5)	8.4 ± 0.8 (-0.4 ± 0.6)
Mouse	0.3 ± 0.2 (0.2 ± 0.3)	0.0 ± 0.2 (-0.1 ± 0.2)	0.0 ± 0.1 (0.3 ± 0.2)	0.0 ± 0.3 (0.1 ± 0.1)	0.2 ± 0.7 (-0.8 ± 0.7)
Human Pool	0.0 ± 0.2 (-0.1 ± 0.2)	0.1 ± 0.1 (0.5 ± 0.3)	0.1 ± 0.1 (0.3 ± 0.2)	-0.3 ± 0.2 (-0.3 ± 0.2)	2.1 ± 0.6 (-0.3 ± 1.0)
Human (1)	0.0 ± 0.4 (0.0 ± 0.3)	0.1 ± 0.2 (0.4 ± 0.3)	0.4 ± 0.5 (0.7 ± 0.6)	0.0 ± 0.4 (0.1 ± 0.1)	0.6 ± 0.7 (0.4 ± 0.3)
Human (2)	0.2 ± 0.5 (0.3 ± 0.2)	0.2 ± 0.1 (-0.2 ± 0.3)	0.3 ± 0.2 (0.1 ± 0.4)	-0.5 ± 0.2 (-0.6 ± 0.3)	1.0 ± 1.1 (-0.3 ± 0.7)
Human (3)	-0.3 ± 0.1 (-0.1 ± 0.2)	0.1 ± 0.3 (0.6 ± 0.2)	0.4 ± 0.2 (1.0 ± 0.3)	-0.4 ± 0.3 (-0.8 ± 0.2)	1.0 ± 1.0 (-0.6 ± 0.9)
Guinea-pig	0.3 ± 0.5 (0.0 ± 0.5)	0.1 ± 0.3 (-0.1 ± 0.2)	0.2 ± 0.2 (0.1 ± 0.2)	0.5 ± 0.2 (0.5 ± 0.1)	1.1 ± 0.4 (-0.1 ± 0.3)
					40.7 ± 4.7 (0.1 ± 0.7)

* Final dilution of serum ---1/5.

+ XMMCO-791 and anti-791T/48 monoclonal antibodies were tested at a final concentration of 125 µ/ml.

++ W6/32 monoclonal antibody was tested at a final dilution of 1/500 of the serum/ascites fluid.

Figures in parenthesis represent % cytotoxicities determined in the presence of heat-inactivated complement.

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H. Conjugation of XMMCO-791 With Ricin Toxin A Chain (RTA)

The conjugation of RTA to an immunoglobulin has been thoroughly described in United States Patent No. 4,590,071, the disclosures of which are hereby incorporated by reference. U.S. Patent No. 4,590,071 fully discloses the technique for RTA purification employing anti-ricin B chain antibodies as well as the conjugation methodology.

I. Characterization of the Immunotoxin

XMMCO-791-RTA was characterized biochemically by SDS-PAGE and functionally by flow cytometry measurement of binding to colorectal carcinoma derived C170 cells and to control MOLT-4 cells (A.T.C.C. Accession No. CRL 1582). It was also characterized by assays that either measured its ability to inhibit protein synthesis in a cell-free system or to kill 791T cells.

In order to determine lot to lot variation, three replicate research lots of immunotoxin were made from the same lot of XMMCO-791 and the same lot of RTA. The conjugations and purifications were done separately but simultaneously. These lots, 51016 A, B, and C, are compared within each section to show lot to lot variation and preliminary data on stability. They have been analyzed by SDS-PAGE, flow cytometry, reticulocyte lysate and whole cell cytotoxicity assays at various times after preparation. The data indicate that the lot to lot variation is minimal, and that the immunotoxins are stable for a least three months.

Results from the assays on a production lot of immunotoxin are also presented in each section. Research lots are prepared in small quantities, whereas production lots are prepared in larger batches under aseptic clean room conditions. The conjugation procedure is the same, but scale up production often produces an increased degree of conjugation. This can be decreased by adjustment of the N-succinimidyl

(2-pyridyldithio) propionate (SPDP) ratio. All lots were very similar, both functionally and biochemically.

1. SDS-PAGE Analysis

a. The Assay

SDS-PAGE analysis of immunotoxins run under nonreducing conditions provides information about the amount of free (unconjugated) antibody remaining in the preparation, the mean RTA/MoAb ratio, the presence of unbound RTA in the preparation, and the number of molecular species present. Two gel analyses are run on each immunotoxin preparation. A 10% nonreduced SDS-PAGE was run to determine the presence of free RTA, producing poor discrimination between the free antibody and the RTA-MoAb conjugates that tend to cluster near the top of the gel. However, it allows excellent definition of any free RTA, which when present is seen as two bands at 30 kD and 33 kD. Free RTA generally constitutes less than 5% of the total protein in freshly prepared conjugate and RTA bands are rarely seen.

A nonreduced SDS-PAGE was run in a 3 -7.5% gradient. This allows accurate detection of the molecular species of conjugate present. Free antibody appears as a band at approximately 160 kD, and immunotoxins with 1, 2, 3 or more RTAs per MoAb appear at appropriate locations reflecting their increased molecular weight.

b. Results

SDS-PAGE analyses were performed on the three replicate research lots of XMMCO-791-RTA and on one of the production lots within two weeks of immunotoxin preparation. The amount of free antibody is similar within the replicate lots, ranging from 1 to 2%. This is typical of free antibody values seen in other research lots. Table XIV summarizes the biochemical characteristics of the replicate and production lots. The RTA to IgG ratio ranged from 3.0

TABLE XIV

Percent of Components in Small or
Large Lots of Immunotoxin

	<u>51203¹</u>	<u>51016A²</u>	<u>51016B²</u>	<u>51016C²</u>
5 Free RTA	0	5.3%	5.6%	4.4%
Free MoAB	0	1.1%	2.1%	2.2%
Monomer	0	12	10	10
Dimer	4%	25	21	10
Trimer	18%	30	27	29
Tetramer	32%	21	23	26
Pentamer	46%	8%	12	16
10 Hexamer	--	3%	5	7
RTA:MoAb ratio	4.2	3.0	3.2	3.4

¹ Production lot, yield 1000 mg

² Replicate lots, yield approximately 20 mg

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to 3.4 on the replicat lots. Molecular species corresponding to free MoAb or 1-6 RTA/MoAb were seen; this was similar for all research lots. By nonreduced 10% SDS-PAGE, about 5% free RTA was detected.

The production lot, 51203, was used for stability testing, and the results of these tests at one and two months are shown in Table XV, indicating that the product is stable. Four molecular species were detected on nonreduced gradient gels, corresponding to dimers, trimers, tetramers and pentamers. There was no free antibody and no band corresponding to monomers. Nonreduced 10% gels showed that minimal free RTA was present.

c. Summary

Both research and production lots showed little free RTA when freshly prepared. The research lots had up to 1-3% free antibody and molecular species corresponding to 1-6 RTA/MoAb, whereas the production lot had no detectable free antibody and no detectable monomers. Molecular species of up to five RTA/MoAb were present in the production lot. Little deterioration was noted over 3 months.

2. Flow Cytometry Studies, XMMCO-791-RTA Binding to C170 Cell Line

a. The Assay

The competitive inhibition assay, by which flow cytometry is utilized to determine binding of unlabeled XMMCO-791 to C170 colon carcinoma cells, is known to those skilled in the art. This assay is particularly well-suited to assess retention of antibody binding in

TABLE V-6

Change in Composition of Replicate Lots
of XMMCO-791-RTA With Time

	<u>51016 A</u>		<u>51016 B</u>		<u>51016 C</u>	
	Initial	3 mo	Initial	3 mo	Initial	3 mo
5 Free RTA	5.3	2.9	5.6	3.7	4.4	2.6
Free MoAB	1.1	4.3	2.1	3.3	2.2	1.8
10 Monomer	12	18	10	15	10	13
Dimer	25	28	21	26	10	27
Trimer	30	27	27	31	29	27
Tetramer	21	23	23	25	26	31
15 Pentamer	8	0	12	0	16	0
Hexamers	3	0	5	0	7	0
RTA:MoAb Ratio	3.0	2.5	3.2	2.6	3.4	2.7

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drug antibody conjugates such as immunotoxins because it measures the ability of unlabeled antibody to specifically compete with FITC-labeled homologous antibody for antigen binding sites.

The reference curve was generated by mixing unlabeled XMMCO-791 standard with FITC-XMMCO-791 and reacting it with C170 cells. This was the positive control. Negative controls included an unlabeled antibody that does not bind to C170 and thus cannot compete. The slope of this line is zero.

The immunotoxin was evaluated by comparing it with its "parent lot" - the MoAb from the same lot used for RTA conjugation. Thus, two sets of mixtures were prepared, the first being a mixture of MoAb from the immunotoxin parent lot with the standard FITC-MoAb and the second, a mixture of the immunotoxin with the FITC-MoAb. When the mixtures were added to C170 cells and assessed for fluorescence intensity by flow cytometry, two lines were generated. Comparison of the slope of the line generated by the parent MoAb with that of the immunotoxin describes the degree of activity retained by the immunotoxin. Since the assay is highly concentration dependent, it was necessary to correct the concentration of the immunotoxin so that it was expressed in terms of antibody concentration instead of immunotoxin concentration.

25 b. Results

Both the replicate lots had RTA to MoAb ratios of 3-3.4 RTA:MoAb; the more highly conjugated production lot had 4.2. The lots tested for binding at various times after conjugation. The results are shown in Table XVI, and are expressed either as amount of unlabeled antibody necessary to decrease fluorescence by 50% (IF_{50}) or as percent decrease in line slope. By the latter parameter, it can be seen that there was little difference in the replicate lots, all of which bound about 80% of their parent lot. Moreover, there

was little noticeable deterioration, and testing three months after production still revealed binding that was greater than 80% of the parent lot. Table XVI also shows the percent binding of the production lot 51203, which had a higher RTA:MoAb ratio than the smaller lots and bound 48% of the parent lot. There was no decrease in binding two months after conjugation.

c. Summary

Replicate lots made under the same conditions showed uniform binding and little loss of activity compared to unconjugated parent lot. There was no apparent decrease in binding over a three-month period. This demonstrates that RTA:MoAb ratios of 3:1 produce little loss in activity.

The first production lot had an RTA:MoAb ratio of 4.2:1 and decreased binding to about 50% of the unconjugated parent lot. Although this had little effect on either the whole cell cytotoxicity assay, the xenograft therapy trials, or the toxicity, we feel that this represents the upper limit of desirable conjugation. In spite of the higher degree of conjugation, there was no apparent decrease in binding over a two-month period.

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TABLE XVI

Binding by Flow Cytometry of Lots of XMMCO-791-RTA
Assayed at Various Times After Conjugation

5	Lot	Time After Conjugation	IF ₅₀ test		Ratio of Test Slope To Parent Slope
			IF ₅₀	Parent	
10	51016A	2 Weeks	-		
		1 Month	77%		79.8%
		2 Month	82%		89.1%
		3 Month	79%		83.9%
15	B	2 Weeks	-		
		1 Month	79%		81.9%
		2 Month	88%		95.3%
		3 Month	80%		88.3%
20	C	2 Weeks	-		
		1 Month	80%		84.0%
		2 Month	81%		86.3%
		3 Month	77%		83.0%
2	51203	2 Weeks	45%		47.7%
		1 Month	50%		52.2%

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3. Reticulocyte Lysate Assay

a. The Assay

This assay measured the ability of XMMCO-791-RTA to inhibit protein synthesis in a cell free system, and compared it with the inhibition produced by free RTA. Although both bound and free RTA should theoretically produce the same amount of inhibition, there is usually a slight loss of activity in the conjugate when the two compounds are compared on the basis of RTA molarity.

Rabbit reticulocyte lysate is prepared by lysing rabbit reticulocytes (precursor red blood cells) in water. A nutrient solution is prepared consisting of standard preparations of all amino acids (except leucine), ^{14}C -leucine, salts, EDTA, hemin (enzyme co-factor), and creatine phosphokinase (the enzyme which catalyzes translation). This solution contains all materials necessary for protein synthesis.

Dilutions of RTA or RTA-containing products were added to types containing the nutrient solution and the lysate. The incorporation of ^{14}C -leucine into protein by the lysate was measured to determine the degree of inhibition of protein synthesis. Results are expressed as a percentage compared to non-RTA containing tubes (100% reference).

Values are expressed as gm/ml producing 50% inhibition of protein synthesis (IC_{50}). The assay may vary by ± 2.5 fold in replicate analysis.

b. Results

Table XVII shows assays on the three replicate immunotoxin lots and one production lot. When plotted in terms of gm/ml, the IC_{50} values of the replicate lots ranged between 2.55 and 3.8×10^{-9} . Free RTA gave an IC_{50} of about 3×10^{-10} gm/ml. Within the limits of the assay, the lots with RTA:MoAb conjugation ratios of 3-3.4 gave the same IC_{50} values. The IC_{50} of

the lot with the higher RTA:MoAb conjugation ratio was lower, although still within the range of five fold.

In order to determine if RTA activity was lost after conjugation to MoAb, these same values were expressed in terms of equivalent RTA molarity. Some loss of RTA activity with conjugation was seen in the
5 lots with RTA:MoAb ratio of 3, and more loss was noted in the lot with a RTA:MoAb ratio of 4. Other immunotoxins tested show no loss of RTA activity at conjugation ratios of 3.0. This suggest that the con-
jugated RTA remains biochemically active, but with
10 higher conjugation there is steric hindrance of its activity.

c. Summary

The RLA assay demonstrates that little loss in activity of RTA is seen after conjugation. Since
15 the production lot of immunotoxin had a higher conjugation ratio than the research lots, the IC_{50} in terms of gm/ml was different. When expressed in terms of RTA molarity, however, the values are similar, although the lots with lower conjugation ratios have higher relative
20 activity.

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TABLE XVII

Reticulocyte Lysate Assay: IC_{50} of Several
Lots of XMMCO-791-RTA

5	Lot#	RTA:IgG Ratio	Immunotoxin (gm/ml IC_{50})	Equivalent RTA Molarity	
				Immunotoxin* IC_{50}	RTA Standard IC_{50}
	51016A	3.0	3.85×10^{-9}	51 pM	5 pM
	51016B	3.2	2.55×10^{-9}	35 pM	5 pM
10	51016C	3.4	2.8×10^{-9}	41 pM	5 pM
	51203	4.2	1.5×10^{-8}	134 pM	8.1 pM

* Calculated by multiplying the molarity of immunotoxin IC_{50} by the number of RTA moieties per MoAb; e.g., Lot 51016A has an IC_{50} of 11 pM and three RTA moieties/MoAb. Thus the equivalent RTA molarity is 51 pM.

The three replicate lots were reanalyzed three months after preparation and the results are shown in the following Table. Little loss of RTA activity is seen.

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TABLE XVIII

Time after Production
IC₅₀ gm/ml

		2 Weeks	3 Months
5	51016A	3.8×10^{-9}	3.3×10^{-9}
	51016B	2.55×10^{-9}	3×10^{-9}
	51016C	2.8×10^{-9}	5.8×10^{-9}

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4. Whole Cell Cytotoxicity Assay

a. The Assay

This assay uses 791T as a positive cell line and MOLT-4 (A.T.C.C. Accession No. CRL 1582) as a negative cell line. It was designed to assess the ability of XMMCO-791-RTA to specifically kill cells expressing the gp72 antigen and is expressed as concentration of immunotoxin producing 50% inhibition of thymidine uptake. MOLT-4, a malignant T cell line lacking the gp72 antigen, is used as the negative cell line.

There are several tumor derived cell lines that carry the gp72 antigen and are specifically killed by XMMCO-791-RTA, as determined using various assays, all of which measure decreased protein synthesis (^{75}Se -Selenomethionine) or cell division (thymidine incorporation). Cell lines carrying the gp72 antigen include the colon carcinoma derived cell line C170, used in the flow cytometry analysis of various immunotoxin lots, and colon carcinoma derived cell line LS174.

791T cells or MOLT-4 cells were grown in serum-free medium for 4 to 7 days in T75 tissue culture flasks. 791T, an adherent cell line, was harvested using trypsin-EDTA (Gibco Labs) because it leaves the gp72 antigen intact. MOLT-4 is a suspension cell line. Cells were adjusted to the appropriate concentration, and the concentration used was related to the cells' rate of growth.

For convenience, the assay was carried out on cells in suspension. To prevent 791T cells from adhering during the assay, cells were plated into non-tissue culture coated NUNC 96-well round bottom plates, 100 μl /well to a final concentration of 2×10^4 cells/well (791T) or 4×10^4 cells/well (MOLT-4).

Immunotoxin dilutions were prepared using test tubes precoated with BSA. One hundred μl of each dilution were added to four wells containing cells, for

final concentrations of 2.5 to 250,000 ng/ml. The RTA was diluted in a similar fashion to yield final concentrations of 500 µg/mg to 50µg/ml. After a 48-hour incubation at 37°C and 5% CO₂, 1 µCi of ³H-Thymidine was added to each well. After another 24-hour incubation, cells were harvested on filter pads and counted.

5 b. Results

Each assay of a lot included testing of the immunotoxin against 791T and MOLT-4 cells. Additionally, free RTA was sometimes tested against 791T cells. When the IC₅₀ values of immunotoxin or free RTA against 791T cells were compared in terms of RTA molarity, the IC₅₀ of the immunotoxin was more than three logs (Log₁₀) that of the free RTA. Also, there was a 3-log₁₀ difference between IC₅₀ of XMMCO-791-RTA immunotoxin against 791T and MOLT-4 cells. The IC₅₀ of XMMCO-791-RTA on negative control MOLT-4 cells (in RTA equivalents) was very similar to that of free RTA on 791T cells.

Table XIX summarizes the IC₅₀ of the replicate and production lots in terms of nanograms/ml and molarity of RTA. Although the IC₅₀ values were quite similar, there was a loss of potency as the conjugation ratio increased, reflected by a higher IC₅₀.

20 c. Summary

The whole cell kill assay measures the potency of intact immunotoxin. There was minimal lot to lot variability, as shown by the results with the three replicate lots. Even though the production lot had a decrease in binding due to the higher conjugation ratio, the IC₅₀ was still similar to those of the replicate lots. This may mean the decreased binding was somewhat compensated for by the increased number of RTA molecules. These IC₅₀ values were similar to those seen with other immunotoxins.

TABLE XIXCytotoxicity of Various lots of XMMCO-791 (IC_{50})

5	Lot	791T				MOLT-4		
		RTA:MoAb Ratio	ng/ml	Molarity (10^{-11})	Immunotoxin RTA	ng/ml	Molarity (10^{-11})	Immunotoxin RTA
	51016A	3.0	6.9	2.9	8.7	11858	4.9	14700
	51016B	3.2	6.8	2.8	8.9	9614	3.9	12500
	51016C	3.4	9.6	3.8	12.9	12166	4.8	16400
10	51203	4.2	17.2	4.3	18.1	5903	3.5	14600

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J. XMMCO-791-RTA Therapy of Human Tumor Xenografts

This study was performed to determine the therapeutic efficacy of XMMCO-791-RTA against human tumor xenografts expressing the gp72 antigen that is recognized by the XMMCO-791 antibody.

1. Procedure

Nude mice were implanted with tumor derived from an established xenograft according. (minced to small 1mm pieces) Three to four days after tumor implantation, groups of up to 10 mice received immunotoxin by intraperitoneal injection and their tumor growth was compared with that in controls receiving saline. A range of treatment schedules, including daily injections for 20 days and a protocol involving treatment 3 times per weeks were employed.

2. Results

In all, three lots of immunotoxins were evaluated, Lots 41203B, 50903 and 51203.

There were three xenograft tests using two different conjugates. In the first series, XMMCO-791-RTA (Lot 41203B) was tested against osteogenic sarcoma 791T xenografts using various treatment protocols. One test scheduled treatment 3 times weekly at a dose of 4.8 mg/kg intraperitoneally for nine doses (total dose 43 mg/kg). Compared with the control group, this produced a significant inhibition of tumor growth (Figure 1). The most effective response with this preparation was achieved in mice receiving eight doses of 12 mg/kg (total dose 96 mg/kg), administered at 2 to 3 day intervals. This is illustrated in Figure 1c, showing that during the treatment there was a marked inhibition of tumor growth. As expected, tumor growth was observed in the treated group after cessation of treatment. In this respect, the rate of tumor growth in the treated group was comparable to that in the controls, although it was displaced in terms of tumor size. Figure 1d summarizes these

trials and shows a good correlation between total dose of XMMCO-791-RTA and therapeutic response, even though different dosing schedules were used.

The overall toxicities observed with this immunotoxin are summarized in Figure 1e. As illustrated, there was no mortality with doses up to 100 mg/kg.

- 5 The only observed effect was a gradual weight loss as doses escalated from 40 mg/kg to 100 mg/kg.

In the second series of trials using XMMCO-791-RTA (Lot 50903), the treatment schedule was 5 mg/kg daily for 20 days (total dose 100 mg/kg). This
10 protocol produced marked inhibition in growth of sarcoma derived 791T xenografts (Figure 2a) and colon carcinoma derived C170 xenografts (Figure 2b). There was significant toxicity, however, with 3/10 mice dying in both tests.

- 15 Taking into consideration the experience gained with two immunotoxin preparations, a third series of test were carried out using XMMCO-791-RTA (Lot 51203). The immunotoxin was given in 20 daily doses of 3 mg/kg/day (total dose 60 mg/kg); the response elicited
20 against colon carcinoma C170 xenografts demonstrated significant suppression of tumor growth. This is illustrated in Figure 3 which shows growth of colon carcinoma C170 xenografts in immunotoxin-treated mice compared with controls. Furthermore, tumor weights in
25 the immunotoxin treated group were significantly less than those in controls ($T/C = 0.52$ $p < 0.02$) at the completion of the trial.

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EXAMPLE II - XMMCO-228-RTA CONJUGATES

A. Production of Hybridomas

Balb/c mice (Bantin & Kingman, U.K.) were immunized with carcinoembryonic antigen (CEA) derived from a perchlorate extract of a colon carcinoma liver metastasis. The immunization schedule consisted of 10 μ g CEA in complete Freund's adjuvant (CFA) given intraperitoneally on Days 0 and 7, and 20 μ g of CEA (Also in CFA) given intraperitoneally on Days 25, 56 and 63.

Three days after the last antigen boost, spleen cells from an immunized mouse were aseptically removed. Following procedures as outlined elsewhere (Galfre et al (1977) Nature 266:550, which is incorporated by reference), 10^6 spleen cells were fused with 10^6 cells of P3-NSI-Ag4-1 (A.T.C.C. Accession No. TIB-18), a hypoxanthine-methotrexate-thymidine (HMT) sensitive murine myeloma cell line. Using polyethylene glycol (PEG), hybrid cells were placed into 96-well culture plates, (Costar, Cambridge, MA #3596) on medium containing a feeder layer of rat peritoneal exudate cells (2.5×10^3 cells/well). Cells were cultured in Dulbecco's Modified Eagle's Medium (Flow Labs, Irvine, U.K.) containing 15% Fetal Calf Serum (Myoclone, Gibco, Paisley, U.K.) and hypoxanthine (10^{-4} M), thymidine (1.6×10^{-5} M) (both from Sigma, Dorset, U.K.) and methotrexate (10^{-5} M) (Lederle, Hampshire, U.K.).

Within two weeks post fusion, cultures of hybridoma cells were tested for antibody binding to CEA by enzyme immunoassay (EIA). Cultures that were positive were cloned using limiting dilution, plating 1-3 cells/well into 96 well culture plates. Wells containing only one colony were identified by microscopic examination, then tested for reactivity with CEA and normal colon antigen (NCA) by EIA and radioimmunoassay

(RIA), and for reactivity on extranuclear membranes from primary colorectal tumors and normal colon by EIA. The clone designated XMMCO-228 was found to stably secrete immunoglobulin which was determined to be of the IgG2a subclass by solid phase RIA using standard methods. Hybridoma XMMCO-228 is presently on deposit with
5 the American Type Culture Collection (A.T.C.C.), 12301 Parklawn Dr., Rockville, MD 20852, USA. The deposit was made on August 14, 1986, and given A.T.C.C. Accession No. HB 9174.

Balb/c mice (Bantin & Kingman, U.K.), 6-10
10 weeks old, were used to culture the hybridoma peritoneally. Approximately 10^7 hybridoma cells were injected into mice that had been pretreated 3 weeks earlier with 0.5 mls of pristane (Aldridge, Gillingham, Dorset, U.K.) injected intraperitoneally (i.p.). The
15 resultant ascites fluid, collected 3 weeks after injection of the hybridomas, contained on average 5 mg/ml of the antibody as determined by measuring immunoglobulin level according to the method of Price and Baldwin ICRS Med. Sci. (1984) 12:1000-01, which is
20 incorporated by reference.

The antibody in ascites fluid was purified by affinity chromatography using a Sepharose - Protein A column using methods well known by those skilled in the art.

25 The hybridoma was grown in vitro in Dulbecco's Minimal Essential Medium (Flow Labs, Irvine, U.K.) with 10% Fetal Calf Serum (Myoclone, Gibco, Paisley, U.K.) and hypoxanthine (10^{-4} M), thymidine (1.6×10^{-5} M) (both from Sigma, Dorset, U.K.) and
30 methotrexate (10^{-5} M) (Lederle, Hampshire, U.K.) in plastic 300 ml bottles. Cell concentration was 10^5 cells/ml medium over a culturing period of 4-5 days, with a MoAb concentration of 4 μ g/ml medium, and a doubling time of 12 hours. compared to E5).
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B. In Vitro Antibody Binding to Cell Lines Measured
by Flow Cytometry

The binding of XMMCO-228 and XMMCO-791 MoAbs to MKN45 cells was determined by flow cytometry employing methods well known to those skilled in the art and described above. The gastric carcinoma cell line MKN45 expresses both the 72kD antigen with which the XMMCO-791 MoAb reacts and the CEA antigen with which XMMCO-228 reacts. B14/B8 is a MoAb directed against breast cancer, which reacts with the normal color antigen (NCA) subsection of the CEA antigen. Normal mouse serum was used as a control. The results are summarized in Table XX.

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TABLE XX

Binding to MKN45 Cells by Flow Cytometry

	<u>MoAb</u>	<u>Mean Fluorescence Intensity</u>
5	XMMCO-228	987
	B14/B8	790
	XMMCO-791	485
10	Control	15

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C. In Vivo Antibody Binding to Normal Tissues
Measured by Immunoperoxidase

The technique for Indirect Immunoperoxidase staining has been described above. Table XXI is a summary of the results obtained with XMMCO-228 MoAbs in humans:

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TABLE XXIAntibody Binding to Normal Tissues
Measured by Immunoperoxidase

COLORECTAL CANCER: 5/5 intense staining of Ca
90%+ of population staining +)

5 NORMAL COLON: 5/5 intense staining of glandular epith.,
especially intense apical staining

AUTOPSY TISSUE: Negative tissue-

10 Kidney
Pancreas
Muscle
Liver
Skin
Heart
Testis
Spleen
Spinal Cord
15 Stomach
Aorta
Cerebellum
Lymph Node

Questionable (+/-)

20 Lung

Positive-

Esophagus(+ squamous epith)

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NOTE: glucose oxidase method showed that Ab. 228 does not stain
granulocytes. Almost every other antibody that we have
studied that is similar to CEA has stained granulocytes.

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It is significant that XMMCO-228 MoAbs do not bind to granulocytes. Normal colon antigen (NCA) appears on such diverse tissues as normal colon, granulocytes, and progenitor bone marrow cells. Because the CEA antigen has a NCA subsection, many anti-CEA MoAbs also bind the NCA on various normal tissues, which XMMCO-228 MoAbs do not.

D. XMMCO-228 Ricin Toxin A Chain (RTA) Conjugates

As in the case of XMMCO-791-RTA conjugates described above, the conjugation technique, including purification of the A chain of ricin, is disclosed in U.S. Patent No. 4,590,071, the disclosures of which are hereby incorporated by reference.

E. XMMCO-228-RTA In Vitro Cytotoxicity

In vitro cytotoxicity of XMMCO-228-RTA conjugates was determined by the ^{75}Se -Selenomethionine Assay. Single cell suspensions of gastric carcinoma derived MKN45 cells were inoculated with immunotoxin or RTA for 15 minutes, washed and added to flat bottomed microtiter plates at a concentration of 10^5 cells/0.2 ml of immunotoxin or RTA in RPMI medium containing 10% fetal calf serum. Various amounts of immunotoxin were added to each well. Twenty-four hours later, ^{75}Se -Selenomethionine was added to each well. Cultures were further incubated for 16 hours, washed, dried on the bottom of the wells, and the wells were counted in a gamma counter. The results are summarized in Table XXII, expressed in terms of 50% inhibition concentration.

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TABLE VIII

In Vitro Cytotoxicity Against MKN45 Cells

		<u>Agent</u>	<u>IC₅₀ (ng/ml)</u>
5		RTA	2238
		XMMCO-791-RTA	1000
		XMMCO-228-RTA	600
		XMMCO-791-RTA + XMMCO-228-RTA (50%/50% mixture)	560
10		<u>Agent</u>	<u>IC₅₀ (molarity)*</u>
		RTA	$1 \times 10^{-8} \text{M}$
		XMMCO-791-RTA	$3.5 \times 10^{-10} \text{M}$
	15	XMMCO-228-RTA	$1 \times 10^{-10} \text{M}$
		XMMCO-791-RTA + XMMCO-228-RTA (50%/50% mixture)	$3.5 \times 10^{-10} \text{M}$
20	*Adjusted for equivalent RTA molarity		

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These data indicate that when XMMCO-791-RTA and XMMCO-228-RTA are administered in a "cocktail" form, the cytotoxicity is at least additive, and possibly synergistic. They also show the efficacy of immunotoxin (or antibody directed RTA) compared to RTA alone.

5 F. XMMCO-228-RTA Therapy of Human Tumor Xenografts

For each data point, 7-10 nude mice were implanted with LS174T cells, a human colon adenocarcinoma derived cell line (A.T.C.C. Accession No. CL 188). Three days post-implant, the first dose of the treatment compound was administered, followed by 10-20 doses until the schedule dose was completed. The results are summarized in Table XXIII, expressed as a ration of the tumor weight in treated mice divided by the tumor weight in control (untreated) mice. The results, in terms of mean tumor diameter, are summarized graphically in Figure 4.

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TABLE XVIII

Therapy of Human Tumor Xenografts
With XMMCO-228-RTA

5	<u>Expt.</u>	<u>Xenograft Tumor</u>	<u>Treatment Compound</u>	<u>Schedule Dose mg/kg</u>	<u>Response: Tumor Weight Treated Tumor Weight Control</u>
	1	LS 174T	RTA	20	0.70
	2	LS 174T	XMMCO-228	80	1.18
10	3	LS 174T	XMMCO-228-RTA	56	0.36

These results demonstrate the efficacy of XMMCO-228-RTA
in the therapy of human tumor xenografts.

EXAMPLE III - XMMCO-791-RTA AND XMMOC-228-RTA COCKTAILS

A. XMMCO-791-RTA + XMMCO-228-RTA Conjugate Cocktails

A cocktail of XMMCO-791-RTA and XMMCO-228-RTA consists of a mixture of XMMCO-791-RTA and XMMCO-228-RTA, in any ratio, and administered to a cancer cell host in a dose range of about 0.01 mg/kg/day to 20.0 mg/kg/day. The cocktail is administered perenterally, generally by intravenous infusion or intraperitoneal injection in a suitable vehicle, such as phosphate buffered saline or the like.

10 B. In Vitro Cytotoxicity of Conjugate Cocktails

In vitro cytotoxicity of XMMCO-228-RTA conjugates was determined by the ⁷⁵Se-Selenomethionine assay. Single cell suspensions of gastric carcinoma derived MKN45 cells were inoculated with immunotoxin or RTA for 15 minutes, washed and added to flat bottomed microtiter plates at a concentration of 10⁵ cells/0.2 ml of immunotoxin or RTA in RPMI medium containing 10% fetal calf serum. Various amounts of immunotoxin were added to each well. Twenty-four hours later, ⁷⁵Se-Selenomethionine was added to each well. Cultures were further incubated for 16 hours, washed, dried on the bottom of the wells, and the wells were counted in a gamma counter. The results are summarized in Table XXII above, expressed in terms of 50% inhibition concentration.

These data indicate that when XMMCO-791-RTA and XMMCO-228-RTA are administered in a "cocktail" form, the cytotoxicity is at least additive, and possibly synergistic. They also show the efficacy of immunotoxin (or antibody) directed RTA compared to RTA alone.

C. XMMCO-791-RTA + XMMCO-228-RTA Conjugate Cocktail Therapy of Human Tumor Xenografts

For each data point, 7-10 nude mice were implanted with MKN 45 cells, derived cell line. Three

days post-implant, the first dose of the treatment compound was administered, followed by 10-20 doses until the scheduled dose was completed. The results are summarized in Table XXIV, expressed as a ratio of the tumor weight in treated mice divided by the tumor weight in control (untreated) mice.

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TABLE XXIVTherapy of Human Tumor Xenografts
With XMMCO-228-RTA

Expt.	Xenograft Tumor	Treatment Compound	Schedule Dose mg/kg	Response:	
				<u>Tumor Weight Treated</u>	<u>Tumor Weight Control</u>
5					
1	MKN 45	XMMCO-228-RTA	25	0.74	
		XMMCO-228	25	1.1	
2	MKN 45	XMMCO-228-RTA	*	0.56	
		+ XMMCO-791-RTA			
10					

*25 mg/kg each of XMMCO-228-RTA and XMMCO-791-RTA administered
as a mixture.

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These data indicate that when XMMCO-791-RTA and XMMCO-228-RTA are administered in a "cocktail" form, the cytotoxicity is at least additive, and possibly synergistic. They also show the efficacy of a cocktail consisting of XMMCO-791-RTA and XMMCO-228-RTA in treating cancer cells in a host.

5 The present invention provides efficacious, novel compounds and methods for the therapy of various cancer cells. The cytotoxic conjugates are particularly efficacious when administered in cocktail form.

10 Although the present invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A cytotoxic conjugate, comprising a ribosomal inhibiting protein bound to an immunoglobulin that defines an epitope on a tumor associated glycoprotein antigen of about 72 kD m.w., said conjugate having the ability to bind said epitope.

2. The cytotoxic conjugate according to Claim 1, wherein the immunoglobulin is produced by hybridoma XMMCO-791 having A.T.C.C. Accession No. HB 9173.

3. A cytotoxic conjugate, comprising a toxin bound to an immunoglobulin that defines an epitope on carcinoembryonic antigen, said conjugate having the ability to bind said epitope.

4. The cytotoxic conjugate according to Claim 3, wherein the immunoglobulin is produced by hybridoma XMMCO-228 having A.T.C.C. Accession No. HB 9174.

5. The cytotoxic conjugate according to Claim 3, wherein the toxin is a ribosomal inhibiting protein.

6. The cytotoxic conjugate according to either of Claims 1 or 5, wherein the ribosomal inhibiting protein is a lectin A chain purified with anti-lectin B chain antibodies.

7. The cytotoxic conjugate according to Claim 6, wherein the lectin A chain is ricin A Chain purified with anti-ricin B chain antibodies.

8. The cytotoxic conjugate according to Claim 6, wherein the lectin A chain is abrin A Chain purified with anti-abrin B chain antibodies.

9. A cytotoxic therapeutic cocktail comprising:

5 a first conjugate of a toxin bound to an immunoglobulin that defines an epitope on a tumor associated antigen of about 72 kD m.w.; and

10 a second conjugate of a toxin bound to an immunoglobulin that defines an epitope on carcinoembryonic antigen.

10. The cytotoxic cocktail according to Claim 9, wherein the immunoglobulin of the first conjugate is that produced by hybridoma XMMCO-791 having A.T.C.C. Accession No. HB 9173.

11. The cytotoxic cocktail according to Claim 9, wherein the immunoglobulin of the first conjugate is that produced by hybridoma XMMCO-228 having A.T.C.C. Accession No. HB 9174.

12. The cytotoxic cocktail according to Claim 10, wherein the immunoglobulin of the second conjugate is that produced by hybridoma XMMCO-228 having A.T.C.C. Accession No. HB 9174.

13. The cytotoxic cocktail according to Claim 9, wherein the toxin of the first and second conjugates is a ribosomal inhibiting protein.

14. The cytotoxic conjugate according to Claim 13, wherein the ribosomal inhibiting protein is a lectin A chain purified with anti-lectin B chain antibodies.

15. The cytotoxic conjugate according to Claim 14, wherein the lectin A chain is ricin A Chain purified with anti-ricin B chain antibodies.

16. The cytotoxic conjugate according to Claim 14, wherein the lectin A chain is abrin A Chain
5 purified with anti-abrin B chain antibodies.

17. The cytotoxic conjugate according to Claim 14, wherein the lectin A chain of the first conjugate is ricin A chain purified with anti-ricin B
10 chain antibodies and the lectin A chain of the second conjugate is abrin A chain purified with anti-abrin B chain antibodies.

18. The use of the cytotoxic conjugate according to claim 1 in preparing compositions for the treatment
15 of cancer.

19. The use of the cytotoxic conjugate according to claim 3 in preparing compositions for the treatment of cancer.

20. The use of the cytotoxic cocktail according to claim 9 in preparing compositions for the treatment of
20 cancer.

21. The use according to either of claims 18 or 19, wherein the therapeutic dose of the cytotoxic conjugate is approximately 0.01 mg to 20.0 mg per kg of host
25 body weight per day.

22. The use according to either of claims 18 or 19, wherein the therapeutic dose of the cytotoxic conjugate is approximately 0.05 mg to 5.0 mg per kg of host body weight per day.

5 23. The use according to claim 20, wherein the therapeutic dose of the cytotoxic cocktail is approximately 0.01 mg to 20.0 mg per kg of host body weight per day.

10 24. The use according to claim 20, wherein the therapeutic dose of the cytotoxic cocktail is approximately 0.05 mg to 5.0 mg per kg of host body weight per day.

25. The use according to any of claims 18, 19, or 20, for the treatment of colon carcinoma.

26. The use according to any of claims 18, 19 or 20 for the treatment of ovarian carcinoma.

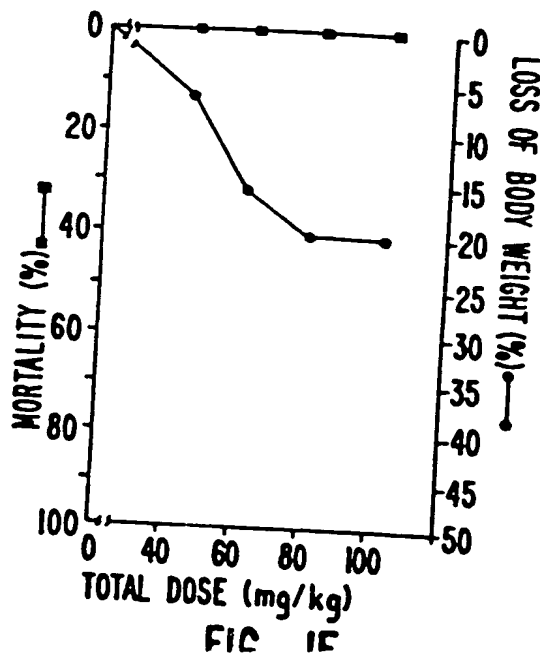
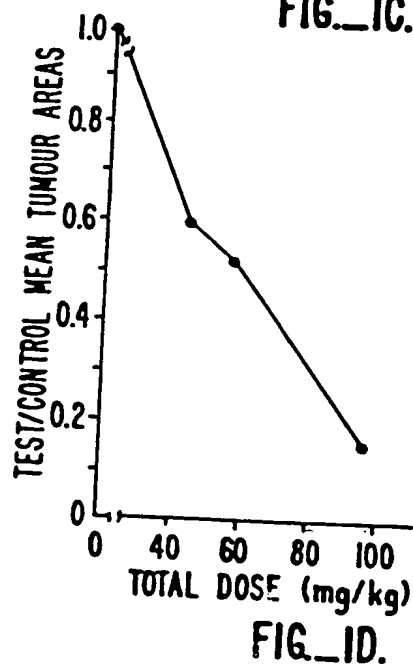
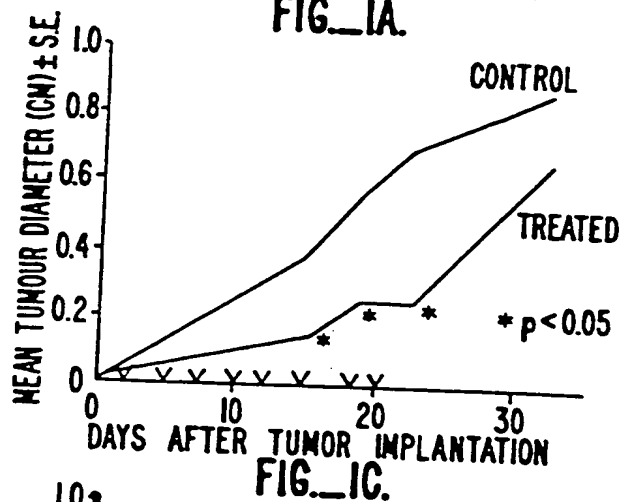
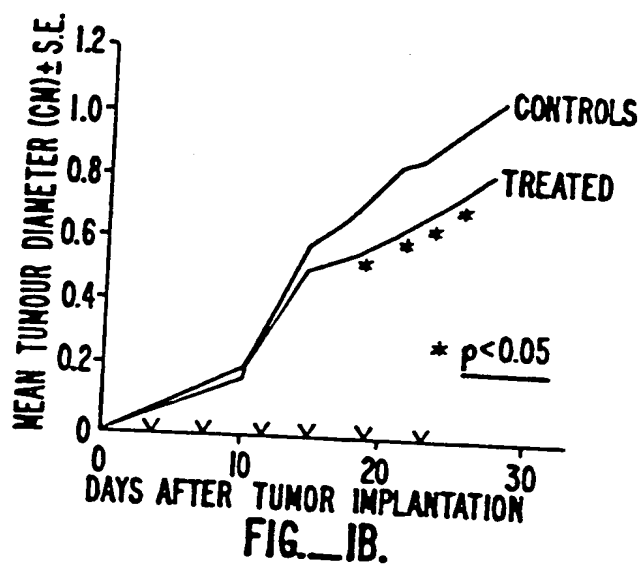
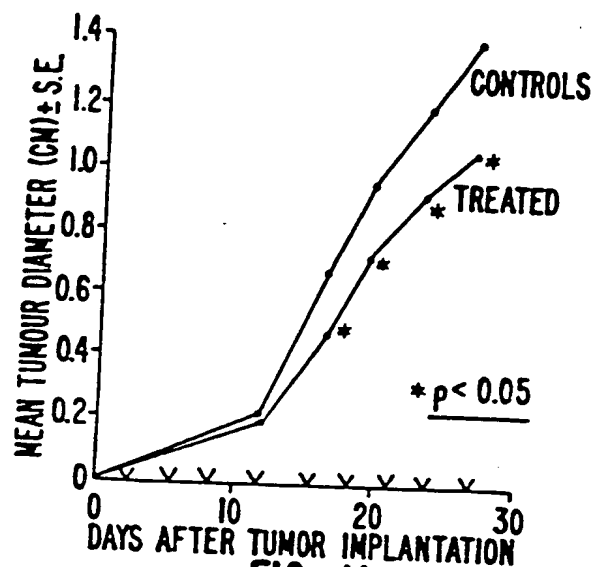
5 27. The use according to any of claims 18, 19, or 20 for the treatment of osteogenic sarcoma.

28. Hybridoma XMMCO-228 having A.T.C.C. Accession No. HB 9174 which produces an immunoglobulin that defines an epitope on carcinoembryonic antigen.

0 29. A kit for the treatment of cancer cells, comprising compositions according to any of claims 1, 3 or 9.

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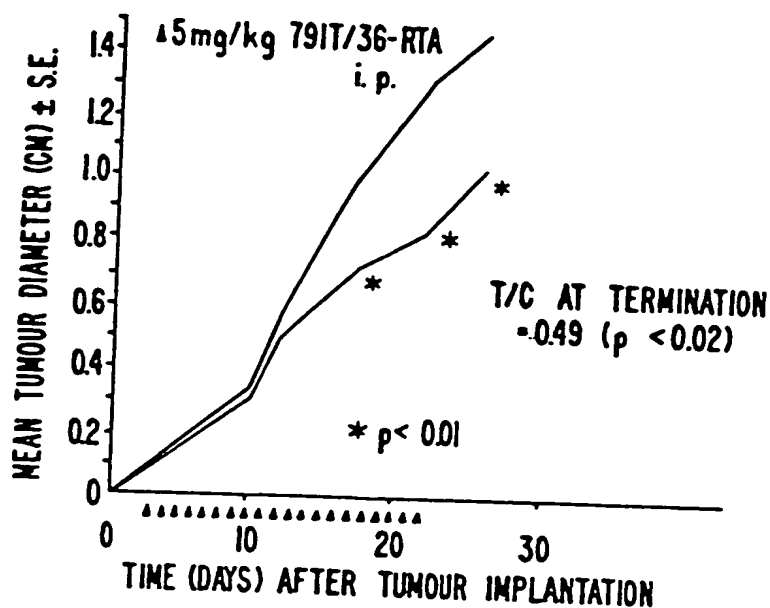
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THERAPY OF MICE BEARING XENOGRAPHS OF SARCOMA 79IT
WITH XMMCO-79I-RTA

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	TUMOUR WEIGHTS ON DAY 25 (mg)	
	79I	C170
TREATED	276	580
CONTROL	925	1180
T/C	0.3	0.49
MORTALITY	3/10 $p < 0.001$	3/10 $p < 0.02$

FIG. 2.

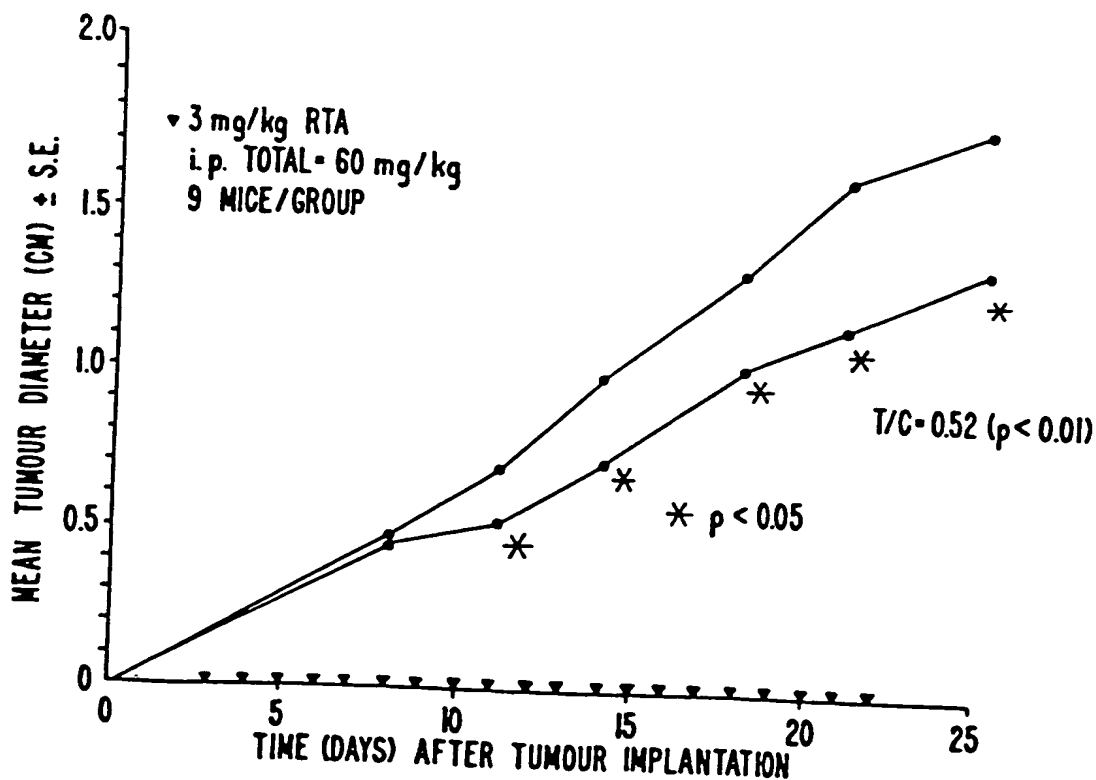


FIG. 2

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INFLUENCE OF RTA-228 ON GROWTH OF LS174T XENOGRAFTS

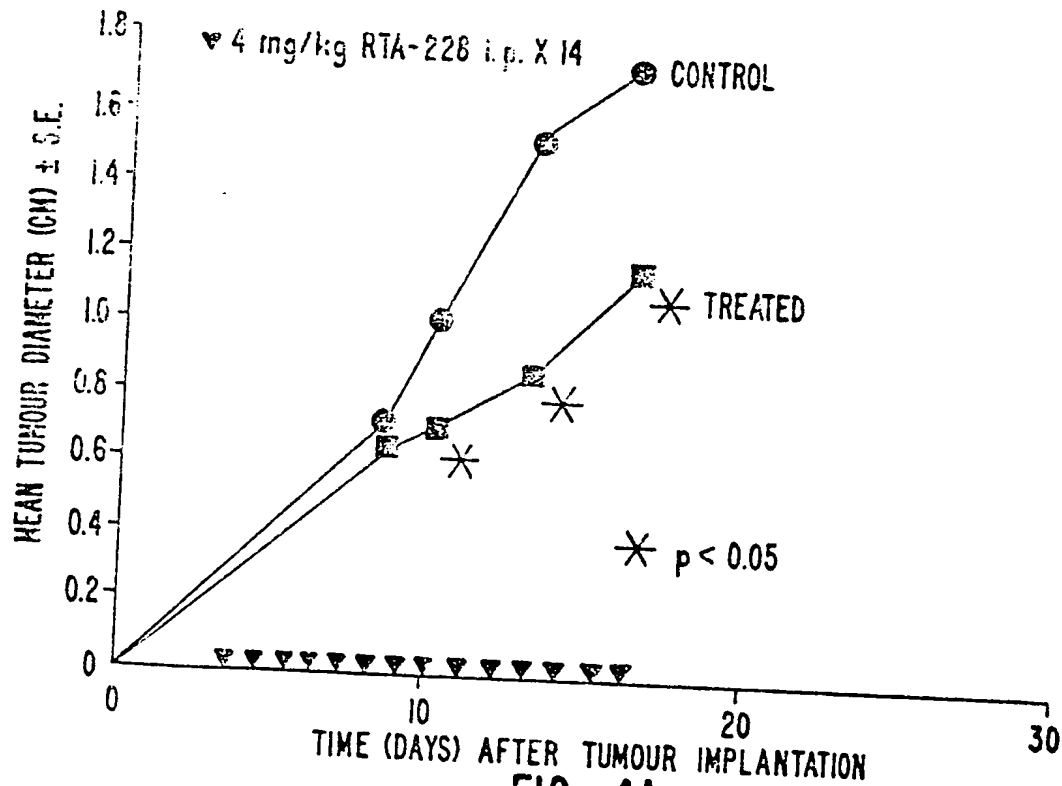


FIG. 4A.

INFLUENCE OF FREE RTA ON GROWTH OF LS174T XENOGRAFTS

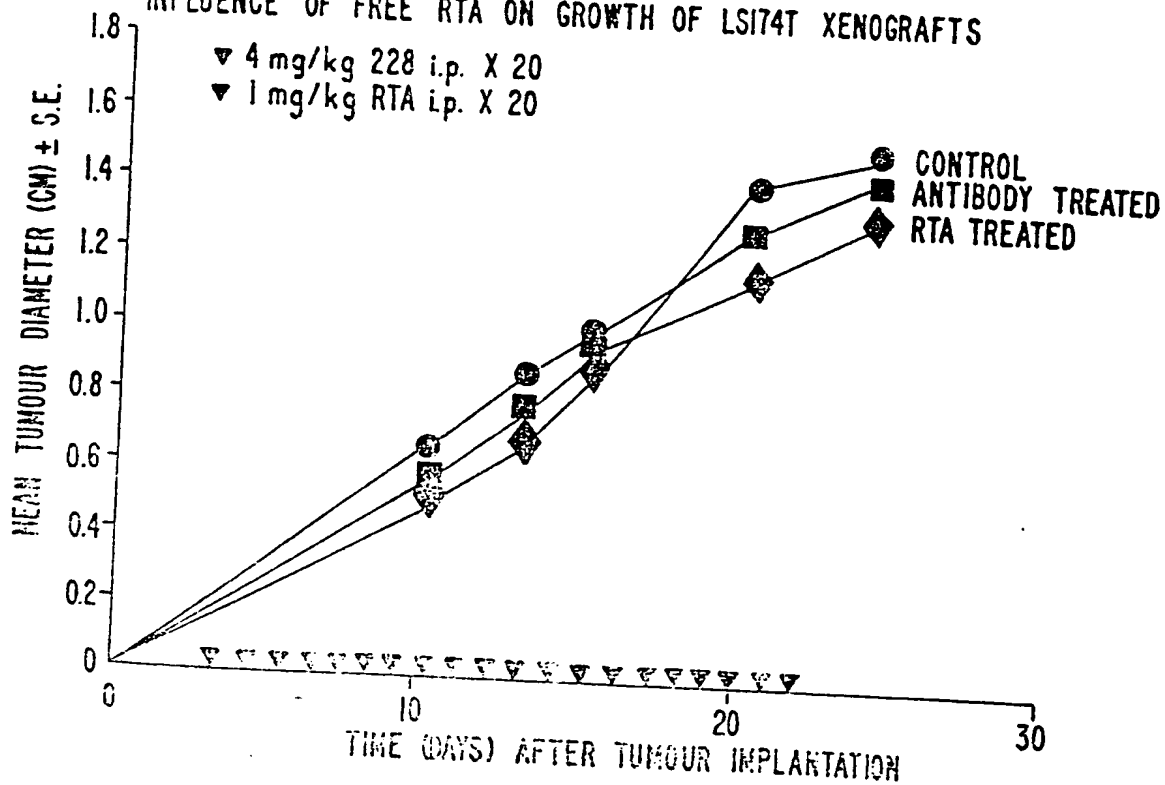


FIG. 4B